LABORATORY DIAGNOSTIC METHODS

IN THE

ARMED FORCES

1963

VOL. III

Issued under the authority of the

DIRECTOR GENERAL,

ARMED FORCES MEDICAL SERVICES

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PART I

HAEMATOLOGY

HAEMATOLOGY

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COLLECTION OF BLOOD

Blood can be withdrawn from either a vein or skin puncture. Venous blood, however, is to be preferred for most haematological examinations. "Capillary" blood is suitable for "screening" when only a few drops of blood are required. Unless a free, spontaneous flow of blood is ensured from a skin puncture, the examination of capillary blood is liable to give variable results.

1. Capillary blood

In the adult the common sites for skin puncture are fingers and lobes of ears. The former is preferred for its easy accessibility and better flow of blood. In an infant, heel, great toe or thumb may be used for puncture. The skin should be warm with free circulation of blood; if cold and clammy, it may be warmed by immersion in warm water for a few minutes. It is a good practice to keep the required apparatus viz. pipettes, slides, dilution fluids ready on the work table, and fix the order of various operations in one's mind before the finger is pricked. The skin is cleaned with alcohol and wiped dry. The sides of the finger are pressed so as to raise the ball of the finger. The ridge so created is pricked with a needle. A bayonet-pointed skin cutting needle struck in a cork and kept immersed in alcohol serves well for the purpose. Use of pins, injection needles or sewing needles should be avoided. The prick should be bold and about 3 mm deep. Light pressure on the sides of this finger in an outward direction helps the flow of blood by opening the wound. Squeezing is to be discouraged. It leads to dilution of capillary blood with tissue juice and thus to erroneous results.

The first drop of blood from a skin puncture is wiped dry with dry cotton wool and subsequent drops of blood are utilized for tests. The pricking needle should be flamed or boiled after use to prevent transmission serum hepatitis.

2. **Venous blood**

This is obtained from ante-cubital vein by a syringe. A dry all-glass paraffin lined syringe or a syringe with metallic piston and No 19-20 SWG needle is used. For preparation of syringes see appendix. Only light pressure on the arm should be applied to make the vein prominent. After withdrawing blood, the needle is detached from the syringe and the blood poured into a container containing a suitable anticoagulant. The container is gently shaken for thorough mixing of anti-coagulant with blood.

Anticoagulants—For preparation of anticoagulant bottles see appendix.

(i) Ammonium and potassium oxalate mixture (Wintrobe's mixture) is the most commonly used. It prevents haemolysis and keeps corpuscular volume unchanged. It is unsuitable for making blood films as it produces crenation of red corpuscles and vacuolation in leucocytes with passage of time. Fragility of red cells, BSR, corpuscular volume, peroxidase test and Van den Bergh's reaction must be done within 3 hours of collection whereas haemoglobin and cell counts can be done even upto 24 hours after collection. Effective concentration of this mixture is 2 mgm per ml of blood.

(ii) Heparin is good for preventing haemolysis and hence is preferred for red cell fragility tests. Effective concentration is 0.2 mgm per ml of blood.

(iii) Sodium citrate is a good anticoagulant for coagulation studies and estimation of sedimentation rate. It is used as 3.8 per cent aqueous solution, one volume being sufficient for nine volumes of blood.

(iv) Preservation of venous blood: The cell counts should be performed on venous blood without much delay. However it can be stored for short durations in a refrigerator at 4°C. Stored samples of blood must be thoroughly shaken for at least two minutes before any cell count is made on these.

ESTIMATION OF HAEMOGLOBIN

Haemoglobin can be estimated with considerable accuracy by finding its oxygen combining capacity or its iron content. However, both methods are tedious to perform and therefore, are unsuitable for routine clinical haemoglobinometry which demands technical simplicity. Haemoglobin pigment and many of its derivatives are intensely coloured materials which are convenient media for colorimetry. Large number of methods have been described but each gives different results and thus arises the need for adopting a single reliable standard method. The chief difficulty encountered in haemoglobinometry is the preparation and calibration of a standard which should remain stable for a long time. Some of the important pigments used are oxyhaemoglobin, carboxyhaemoglobin, reduced haemoglobin, acid and alkaline haematin, cyanhaematin, cyanmethaemoglobin and pyridine haemochromogen. Out of these carboxyhaemoglobin and cyanmethaemoglobin are the most stable and thus are suitable permanent standards. The principle underlying all

colorimetric methods is to convert haemoglobin into another pigment derivative and match its colour against that of a *standard*. The standard is usually prepared from the same pigment but artificial standards in the form of coloured solution or nonfading tinted glass are also sometimes employed. The matching may be done with direct vision, with a photometer or with a photoelectric colorimeter. Three methods will be described viz. Sahli's acid haematin method, oxyhaemoglobin method and cyanmethaemoglobin method.

3. **Sahli's acid haematin method**

(i) Fill the graduated tube of Sahli's haemoglobinometer with N/10 hydrochloric acid to mark 10.

(ii) Draw blood into pipette upto mark 20 (=0.02 ml).

(iii) Without delay pour this blood into acid contained in the graduated tube and remove the last traces of blood by drawing solution into the pipette and then expelling it three times.

(iv) Wait for 40 minutes and then add distilled water drop by drop till its colour matches the colour of the standard.

(v) Now read off the figure on the tube at the bottom of the meniscus. This gives per cent haemoglobin. (Sahli's 100 per cent is equivalent to 14.8 Gram Hb per 100 ml blood).

4. *Sahli-Hellige Haemoglobinometer*—This instrument is available in some military laboratories. It is provided with a glass tube with a square cross section. This is graduated in per cent haemoglobin on one side and Gm per cent of Haemoglobin on the other. In this the colour of the unknown solution is compared with the

standard which consists of nonfading tinted glass. The instrument is calibrated for taking readings for haemoglobin value after 5 minutes action of acid on blood.

5. *Advantages and drawbacks*

The method consists of conversion of haemoglobin into acid haematin and then matching its colour with that of a standard acid haematin solution or tinted glass. Method is highly convenient and hence popular. It has got several drawbacks. Acid haematin is not in true solution and hence precipitation often occurs. Maximum colour takes about 40 minutes to develop and even longer in infants. Final colour is affected by protein, lipoid, bilirubin and carboxy-haemoglobin. Further personal error is considered to be about 10 per cent. In order to get consistent and fairly reliable results, instrument must be standardised at least once a year by comparing with estimation based on iron content. (100 G Haemoglobin contains 0.334 G iron).

3. **Oxyhaemoglobin method**

L *Using grey-wedge photometer*

(i) Draw 0.02 ml of blood into a pipette and pour it into 4 ml of 0.04 per cent (V/V) ammonia contained in a test tube. Mix thoroughly.

(ii) Place this in the glass cell of a photometer.

(iii) Match the density of colour of the test solution in an adjacent half field, against the light transmitted by the rotating grey wedge in the photometer using a yellow-green filter.

Figure on the scale is read off as a percentage (100 per cent=14.8 G Hb per 100 ml).

II. *Using photoelectric colorimeter*

(i) Prepare oxyhaemoglobin solution as above.

(ii) Take a reading of its optical density using a yellow green filter (Denote this reading as R).

(iii) Take a reading of a *standard* oxyhaemoglobin solution made from a blood sample containing 14 G Hb per 100 ml.

(iv) Calculation: $\frac{R}{S} \times 14$=Hb in Gram per 100 ml of blood.

The Oxyhaemoglobin method is easy and rapid but the standard solution tends to fade easily.

7. **Cyanmethaemoglobin method**

(i) Add 0.02 ml blood into 5 ml of Drabkin's solution in a test tube. Stopper the tube by means of a rubber bung and mix the solution thoroughly by inverting several times.

(ii) Allow it to stand for 10 minutes at room temperature.

(iii) Compare it with the standard in a photoelectric colorimeter.

(iv) Standard: Blood containing 5 G, 10 G and 15 G respectively are diluted 1/250 with Drabkin's solution. These are sealed in colorimeter tubes.

(v) *Drabkin's solution*—

$NaHCO_3$	—	1 G
KCN	—	0.2 G
$K_3 Fe(CN)_6$	—	0.2 G

Distilled water to 1 litre.

The method is accurate and the standard solutions last for years, as these do not fade. However, the use of cyanide is a positive disadvantage. For safety, pipettes with bulbs or automatic pipettes should be used.

THE CELL COUNTS

8. **Total red cell count**

I. Place a thick cover slip over the centre of the counting chamber firmly. If it is correctly applied the touching glass surfaces show Newton's multi-coloured and defraction rings.

II. Fill 0.02 ml of blood into a haemoglobinometer pipette, wipe the outside of the lower end of the pipette and expel this into 4 ml of red cell dilution fluid contained in a small bottle or tube and rinse the pipette with the same fluid thrice in order to remove the last trace of blood. Mix thoroughly for 2 minutes.

III. Without delay, charge the counting chamber with this suspension using a Pasteur pipette for the purpose. Take care that the fluid does not flow into the surrounding moat and that no air bubbles get into the fluid.

IV. Allow the fluid in the chamber to settle for 3 minutes.

V. Count all the red cells in 80 small squares (each 1/400 sq mm in area) in the central ruled area with the help of 4 mm dry objective and ×6 or × 10 eye piece. The diagram below marks out areas for red cell and white cell counts. Count should include the cells touching top and right hand margins but omit the cells touching the left hand and bottom margin of the marked squares.

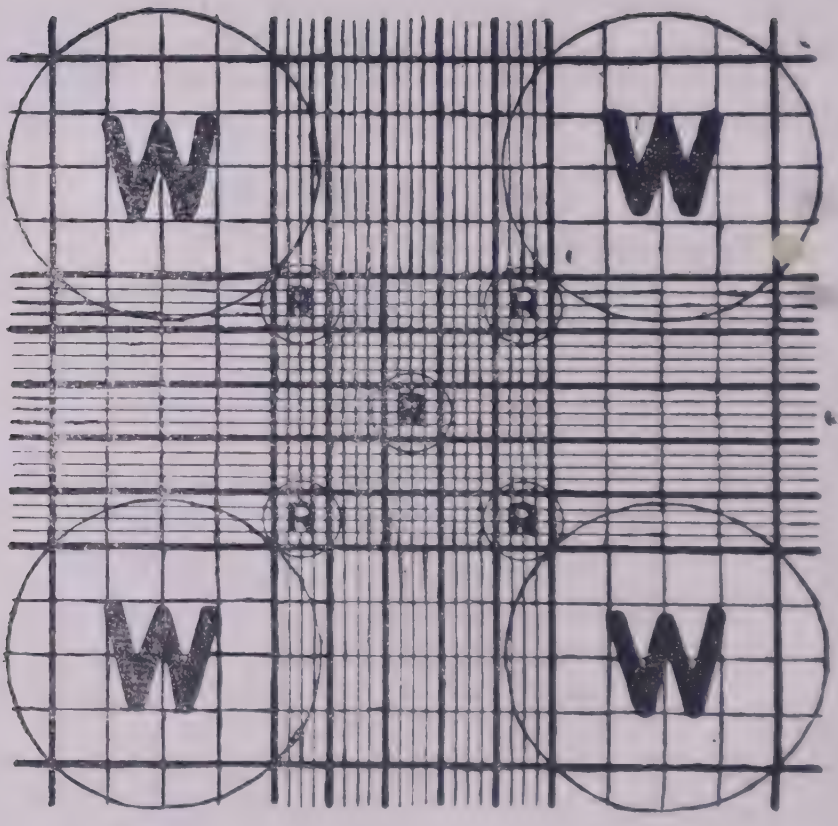


FIG. 1—*Neubauer ruling. Five small circles are used when a red cell count is being done. The large circles denote squares for the WBC count.*

VI. Calculation:
Number of cells in 80 small squares

(area equal to $80 \times \frac{1}{20} \times \frac{1}{20}$ sq mm) 0·1 mm in depth

(volume $= 80 \times \frac{1}{20} \times \frac{1}{20} \times \frac{1}{10} = \frac{1}{50}$ cmm) $= N$

$\therefore$ Red cell count per cmm $= N \times 50 \times 200$
$= N \times 10000$. (200 is the factor for dilution).

Red cell diluting fluid:

Formalin (40 per cent Formald ehyde)—1 ml 3 per cent sodium citrate—99ml

This fluid prevents agglutination of red cells, maintains their normal shape and prevents the growth of moulds.

Errors in the red cell count:

The red cell count is tedious to perform and time consuming. It has a high percentage of error and hence it is important that the technician as well as the clinician should know the limitations of the method. There are several kinds of errors.

(i) "Field error". This is due to the irregular distribution of cells in the chamber. It can be reduced by counting a greater number of cells or by repeating the count.

(ii) Dilution error—This is reduced by bulk dilution of blood as suggested in the method above. The use of red cell counting pipettes is to be discouraged.

(iii) Technical errors—These are due to inaccurate apparatus and faulty technique. The apparatus should be of high quality and checked for accuracy. Technique can be improved by doing the work carefully and without hurry.

An electronic cell counter has been described which gives an accuracy within ± 1 per cent, but the instrument has a prohibitive cost and requires careful calibration.

9. **Total white cell count**

I. Using a white cell counting pipette draw blood upto the 0.5 mark. Wipe the end of the pipette to remove blood sticking to the outer surface of the pipette.

II. Gently suck the diluting fluid into the same pipette upto the mark 11 (eleven).

III. Detach the mouth-piece and rubber tu
and sealing the two ends with the fin
shake the pipette well.

IV. Blow out about one-third of the cont
of the bulb of the pipette.

V. Now with a drop of the fluid charge
counting chamber and allow it to s
for 3 minutes.

VI. Count all white cells in the peripher
large squares of a Neubauer chan
(marked "W" in the diagram on pag
Include cells touching the top and r
hand margins but omit those touching
bottom and left hand margins.

VII. Calculation:
No of cells in 4 large squares (ar
$4\times1\times1=4$ sq mm) 0.1 mm in de
(Volume $4\times0.1=0.4$ cmm)=N.

$$\text{White cell count per cmm} = N \times \frac{1}{0{\cdot}4} \times 20 = N \times$$

White cell diluting fluid:

Glacial acetic acid—1.5 ml
1 per cent gentian violet in water—1 m
Distilled water—make upto 100 ml

Acetic acid lyses red cells and Gentian v
stains white cell nuclei.

Errors in white cell counts:

These are similar to those described u
the red cell count. Nucleated
cells get counted as white cells but
be easily spotted from the stained p
pheral blood film.

10. **Total eosinophil count**

I. Measure 0.02 ml of blood with haemo
binometer pipette and add it to 0.38 m
diluting fluid in a small bottle or t
Mix for ½ minute.

II. Fill a Fuchs-Rosenthal counting chamber with this fluid with the help of a Pasteur pipette.

III. Allow it to stand for 2-3 minutes.

IV. Count all eosinophils in the whole of the ruled area (16 sq mm).

V. *Calculation*:

No. of cells in the whole of the ruled area (Volume $=4\times4\times0.2=3.2$ cmm)$=$N.

$$\text{Total eosinophils per cubic mm} = N \times \frac{1}{3\cdot2} \times 20 \text{ (dilution)}$$

$$= N \times 6\cdot25$$

VI. *Diluting fluid*:

Eosin—0.1 G.
Acetone—10 ml.
Distilled water—100 ml.

This diluent lyses red cells, but inhibits lysis of white cells, especially eosinophils, which are also brightly stained. It is best to fill the chamber soon after diluting the blood and also to avoid prolonged mixing.

11. **Platelet count**

I. Venous blood is collected into a paraffin-lined bottle containing Wintrobe's anticoagulant mixture.

II. 0.1 ml of this blood is drawn into a pipette and this is washed into 9.9 ml of diluting fluid contained in a screw-capped bottle. Mix by inverting several times.

III. Charge the Neubauer chamber with this fluid and keep it in a moist chamber (improvised by putting wet blotting paper at the bottom of a petri dish) for 10 minutes to allow the platelets to settle.

IV. **Examine this now with a 4 mm objec** These appear as highly refractile s pale blue particles. Count all platele 1 sq mm ie one large square of the bauer scale marked "W" in diagram page 8.

V. *Calculation:*

No. of platelets in 1 sq mm (V ime cmm)=N.

Platelets in 1 cmm=N×10×100 (dilut =N×1000.

VI. *Diluting fluid*

Sod citrate—30 G.
Formatin—10 ml.
Dist water—1000 ml.
1 per cent brilliant cresyl blue in nor saline—2 drops.

Always filter before use. This fluid gives g separation of platelets. If clumping occ collect blood with a siliconed syringe and sequestrin as an anticoagulant.

Errors in counts appear to be high in the ha of inexperienced workers, but can be mini ed by careful regard to detail in the techni

STUDY OF DRY BLOOD FILMS

12. Preparation of blood films

These are usually made on glass slides and commonly, on cover slips. In emergenci broken piece of glass may be used: thi examined by mounting it on a regular slide order to get good films it is essential to chemically cleaned glass slides which without scratches or frosting (for clean glassware refer to appendix). For quick cle ing, dip these in glacial acetic acid for minutes, wash with distilled water and t with alcohol. Flame and polish with cl cloth.

I. Place a slide on a firm smooth surface and place a small drop of blood in the middle of the slide about $\frac{1}{2}$" from a narrow edge.

II. Another slide (with the narrow edge made narrower by cutting $\frac{1}{4}$" from both corners) or a cover slip, is used as "spreader". Place the edge of the spreader on the 1st slide at an angle of 45°. Pull it till its back touches the drop of blood which will now spread along the length of the spreading edge.

III. Now take the "spreader" forward with a rapid and uniform movement. The drop will spread into a film.

IV. Dry this immediately by waving it in the air.

V. The name of the patient and the date should be written on the film, with either a lead pencil or a pin (This is not removed by staining or mounting).

VI. Blood films should be stained within a few hours of their preparation, failing which they should be fixed in methyl alcohol for 2 minutes and then dried and stored by wrapping them together, with a layer of paper between slides.

The common fault with films is that they are too thick. Thin films are obtained by reducing the angle of "Spreader" and slowing its movement while making the film. An ideal film should be about $1\frac{1}{2}$" long and $\frac{3}{4}$" wide. It should be slightly thick at its beginning but reasonably thin near its tail, where red cells should be seen, just touching one another but not overlapping. When held against light, the thin portion of the film should show a play of colours.

Films which are too long or too wide, which show bands due to irregular thickness, vacuoles due to grease, or streakiness due to an irregular edge to the "Spreader" should be rejected.

13. **Staining methods**

Romanowski stains are commonly employed for staining blood films. These are prepared by compounding methylene blue and eosin in different proportions and with various treatments. An important compound, thus formed, is methylene blue azure which stains azurophyl granules of leucocytes. Acidic groupings of nucleic acids in nuclei and proteins in primitive cytoplasm tend to combine with methylene blue and its derivatives, whereas basic groupings of haemoglobin combine with acidic dye eg. eosin. It should, therefore, be realised that these stains will be sensitive to the pH of solutions used in their dilution. in the washing of the slide after staining: the pH of the oil used for the oil immersion also matters (A pH of 6.8 is recommended for this purpose). (Refer to appendix for preparation of buffers).

14. *Leishman's stain*: (For preparation see appendix)

(i) To the dry blood film add one volume of Leishman's stain with a 'marked' Pasteur pipette so that the stain fully covers the film. Allow to stain for $\frac{1}{2}$—1 minute.

(ii) Pour two volumes of buffered distilled water (pH 6.8) with a separate marked Pasteur pipette. Mix this with the stain by gently blowing over the slide. Allow to stain for 7 to 10 minutes.

(iii) Wash with gentle stream of buffered distilled water. Wipe the back of the

slide and set it upright to dry. The colour of the film should be a bluish pink.

This is a good stain for routine use. The stain is in alcoholic solution, hence the undiluted stain fixes the blood film as well as stains it. Even a trace of water getting into the bottle of stain spoils it; hence, it is essential to keep a Pasteur pipette exclusively for pouring stain. If the film is overstained or is covered with an excessive precipitate of stain, pour a little undiluted stain over it and immediately wash with buffered distilled water.

15. *Giemsa's stain*:

(i) Fix the dry blood film by immersing in methyl alcohol for 3 minutes.

(ii) Place this slide (while still wet with alcohol) with film downwards into a Giemsa staining rack containing a liberal amount of Giemsa stain diluted 1/20 with distilled water.

(iii) Allow to stain for 20 minutes or more.

(iv) Wash in distilled water and dry in the air. Giemsa stain contains a number of compounds of methylene blue and eosin known as Azure I, Azure II and Azure III eosin. It gives good staining for parasites but.red cells and neutrophil granules stain poorly. It is an aqueous stain and its use leads to saving of methyl alcohol.

16. *Wright's Stain*:

(i) Pour undiluted stain over a blood film and keep for 1 minute.

(ii) Add an equal volume of distilled water. Mix by blowing over the stain. Keep for 5 minutes.

(iii) Wash with tap water and dry.

Some batches of stain require the use o 'buffered distilled water' of pH 6.4.

17. *J.S.B. Stain*:

(i) Fix the dry blood film by immersin in methyl alcohol for 1-2 seconds an then dry it thoroughly.

(ii) Immerse in solution II (Eosin) for 1 seconds.

(iii) Remove excess of eosin by dipping i 'buffered water'.

(iv) Transfer it to solution I and keep fo 40—45 seconds.

(v) Wash by dipping in buffered was water for 3-4 seconds.

(vi) Dry the slide.

For staining thick smears, omit step (i).

Advantage of the method are low cost of stai easy preparation, quick staining and goo staining of the blood parasities.

18. *Combined Leishman-Giemsa staining*:

(i) Pour Leishman solution over th dry blood film and keep for 1 minut

(ii) Pour away this stain and cover th smear with diluted Giemsa stain 1/2 and keep for 10 minutes.

(iii) Wash and dry.

This gives brilliant staining of nuclei, leucocy tic granules and parasites and is thus highl suitable for staining all marrow films an peripheral blood smears in certain speci conditions eg Leukaemia, where good stain ing helps correct identification of primitiv cells.

19. ***May-Grunwald-Giemsa Stain*:**

(i) Dry films in air and then fix in methyl alcohol for 10—20 minutes.

(ii) Transfer these to a jar containing May-Grunwald stain (see appendix) diluted with equal volume of buffer (pH 6.8) and keep for 5 minutes.

(iii) Transfer now to a jar containing a freshly made 1/10 dilution of Giemsa stain in buffered distilled water. Keep for 10—15 minutes.

(iv) Differentiate by placing in buffer for 4—12 minutes, the exact time depending on thickness of film and directly controlled by the observation of the wet smear under the low power objective of the microscope.

(v) Dry in the air and mount under a large cover slip using neutral Gurr's medium. This saves the blood film from dust and scratches and the film may thus be well preserved for at least five years.

20. **Differential leucocyte count**

I. Examine a well stained blood film with 1/12th lens under oil immersion, traversing the film in the "battlement" system in an area of smear which is well spread and is 1 cm internal to the tail of the smear.

II. Record the number of each type of white blood cell that is met with. An easy and rapid method of recording is to count neutrophils against the lymphocytes upto a total of 20, making a mark for each of the other types as these come into the field of vision.

III. A count of 200 leucocytes in a well made film will give about ±5 per cent error.

IV. In leucopenic states thick films should be used for a differential count. However, this has the disadvantage that it becomes difficult to distinguish between mononuclear and large lymphocytes.

21. **Reporting on blood films**

This is the most valuable single blood examination, provided the observer has proper training and experience. The film should necessarily be well spread and properly stained. It should be examined methodically. Scanning with low power objective gives an idea of distribution of red cells and leucocytes. Having found a suitable area of the film, one should examine it under the oil immersion lens. The report should be descriptive in nature. The extent of various abnormalities should be expressed as mild, moderate or severe rather than +, ++ etc. The important abnormalities seen in the blood smear are tabulated below. For a more detailed description, reference to a text book of haematology may be made.

Red blood cells:

Size—Anisocytosis, macrocytes, microcytes.

Shape—Poikilocytosis: sickle cells, oval cells, Burr cells, Schistocytes, Spherocytes.

Staining—Hypochromia, polychromasia, Basophilic stippling, Heinz bodies, dense hard staining round cells (spherocytes), target cells.

Nuclear remnants—Cabot's rings, Howell-Jolly bodies.

Nucleated red cells—Normoblasts, megaloblasts.

White blood cells:

Increase or decrease in number.

Primitive leucocytes, toxic granulation of neutrophils, hypersegmented neutrophils.

Platelets:

Abnormal size, lack of granularity and basophilic staining. Increase or decrease in number.

Parasites:

Malarial parasites, LD bodies, microfilaria, spirochaeta recurrentis, spirillum minus.

RETICULOCYTE COUNT

22. *Method*

I. Place two drops of aqueous cresyl blue solution in a Khan tube (1 per cent water soluble brilliant cresyl blue in Citrate-saline solution made by mixing one part of 3 per cent sod citrate and four parts of normal saline).

II. Add four drops of the patient's blood, mix and place in an incubator at 37°C for 15 minutes.

III. Shake the tube to resuspend the cells and make thin smears on ordinary glass slides and dry these.

IV. Examine smear under the oil immersion objective and ocular with an adjustable diaphragm or an improvised diaphagm of cardboard or tin with a square or circular area cut in its middle. Count reticulocytes in relation to total number of normal red cells.

V. Counterstaining is not recommended, as prolonged counterstaining tends to wash away the reticular material and deposit a precipitate of the stain.

VI. The total number of normal red cells to be counted is about 1000 cells or the number counted when 100 reticulocytes have been encountered.

Reticulocytes are young red cells containing small traces of ribonucleoprotein which appear as blue granules or filaments when stained vitally with brilliant cresyl blue.

An increased number of reticulocytes in the peripheral blood indicates bone marrow activity and their number is roughly proportional to the degree of marrow activity.

The reticulocyte count is of value in the diagnosis of concealed haemorrhage, in chronic haemolytic anaemias and lead poisoning. A rise in the reticulocyte count after the administration of a haematinic indicates its efficacy. This reaction is utilised for the standardisation of therapeutic agents eg liver extract.

PACKED CELL VOLUME (P.C.V)

23. *Method*

I. Collect venous blood in a Wintrobe's anticoagulant bottle.

II. Shake the blood to resuspend the cells properly.

III. Fill blood into Wintrobe tube upto 100 mm mark with the help of a Pasteur pipette having a capillary long enough to reach the bottom of the Wintrobe tube.

IV. Centrifuge for 30 minutes at 3000 rev/min.

V. Read the height of red cell column (excluding white and yellow lines of leucocytes and platelets on top of the red cell column) and express it as a percentage of whole blood.

Packed cell volume can be determined within an error of $\pm$ 1 per cent. It is necessary for the determination of "absolute values"

24. *Calculaton of absolute values*

$$\text{Mean cell volume (M.C.V.)} = \frac{\text{P.C.V.} \times 10}{\text{R.B. Cs (in millions/cmm)}} C\mu \text{ (cubic microns)}$$

$$\text{Mean cell haemoglobin concentration (M.C.H.C.)} = \frac{\text{Hb (in Gram/100 cc blood)} \times 100}{\text{P.C.V.}}$$

MCHC is expressed as a percentage.

The other values and indices eg colour index, mean cell haemoglobin, have been purposely omitted as these do not furnish useful information and have become obsolete.

MCV and MCHC form the basis of a morphological classification of anaemias and hence are very useful. However, though the MCHC is always reasonably accurate the MCV is not, on account of the low accuracy inherent in red cell count.

25. **Measurement of red cell diameter**

A simple and direct way using the eye piece micrometer scale is described. The scale is first calibrated (by using a Neubauer Chamber on the stage; one side of WBC count square is 1 mm in length) in relation to oil immersion objective, eyepiece and a fixed tube length. A conversion table is prepared and always kept near the microscope eg using ×6 eyepiece and 2 mm oil immersion objective.

5.0 divisions=6.6 μ

5.5 divisions=7.2 μ and so on.

Measure a few representative cells in the smear and get an idea about the average diameter, or measure about 50 cells and get the arithmetical average size. For measuring diameters, an area of film should be selected where cells are just touching each other and

are well spread. Red cells in dry film a shrunken about 8 to 16 per cent as compare to the wet film. In the thick part of the bloc film, the red cell diameter is significantl smaller.

BONE MARROW BIOPSY

26. Examination of bone marrow is a valuab procedure which has diagnostic importance in mar blood disorders and is often helpful in assessing pr gnosis and deciding on a therapeutic remedy.

Specimen of bone marrow may be obtained eith by a surgical biopsy or a puncture-aspiration. Th latter has almost entirely replaced the former exce rarely in myelofibrosis, osteosclerosis or metastat tumour deposits. Puncture aspiration biopsy is saf simple and comparatively less painful. Surgical biops (trephine), however, has the advantage of showin architectural relationship between different blood cel and taps a bigger area but has a greater risk of ha morrhage and infection. The only definite absolut contraindication for bone marrow biopsy is haem philia.

27. *Technique of puncture aspiration*—The mo favourable site is 1st piece of the body of the sternu but the manubrium and 2nd piece of the body of th sternum are equally suitable. Other sites sometime used are the spinous processes and the iliac crest an these afford sites when punctures have to be repeate or when sternal puncture results in a 'dry tap'.

I. Keep patient on his back, infiltrate ½ ml of per cent Novocaine into skin, subcutaneou tissue and periosteum of sternum opposite 2n intercostal space.

II. A Salah or better a Klima pattern needle, pr viously sterilized, is used. Introduce the needl through the skin till it touches the periosteun

Now adjust the stop at 5 mm above the skin level. Drive the needle through outer table of the sternum, using gentle pressure. A definite 'give' is felt as soon as the needle reaches the marrow cavity.

III. Remove stylet, attach a 2 ml syringe and suck-till about 0.25 ml of marrow has been withdrawn. A peculiar pain is felt if needle is in the proper cavity.

KLIMA
PATTERN NEEDLE

SALAH
NEEDLE FOR
STERNAL PUNCTURE

FIG. 2

IV. Make thin smears from a drop and pour the rest into a narrow tube containing mixture of ammonium and potassium oxalate (see appendix). Make several smears from this within ½ hour of collection.

V. Pour this material on a watch glass. Le
settle for 5 minutes. Tilt the watch glass
pick out marrow fragments with two need
Place these on a slide, tease with needles
then make a thin smear in the routine w
These are called 'Particle Smears'. An al
native procedure is to crush these between
slides, later pulling the two slides apart. T
has the disadvantage of distorting the c
but the method is valuable in conditions
aplasia and myelofibrosis where the archi
ture of the bone marrow and the spatial r
tionship of cells has to be seen.

VI. In the case of hypocellular marrow, be
smears can be made by centrifuging the
lated marrow specimen, and making mar
films from the mixture of the deposit with
equal volume of the supernatant. Staining
marrow films is similar to staining of p
pheral blood films. Fixation of film
methanol for 20 minutes is essential to b
out the best staining.

28. *Reporting on marrow films*

A total and differential cell count of mar
is often done, but this is fallacious and
not give any more information than a qua
tive assessment. This is due to the fact
marrow always gets diluted with periph
blood, and further, the cells of marrow
sticky and tend to adhere in small clumps.
bigger and more primitive cells tend to r
aspiration and the few that appear in
smear are irregularly distributed. Finally
normal range of cell counts in marrow
pretty wide. Several smears should be exa
ed rather than giving an opinion after s
one slide only.

Smears having some marrow granules are adequate, and proportions of different cells should be assessed in the trails of these granules. Examination under the low power objective gives an idea of the cellularity and the number of megakaryocytes can be judged. This is followed by examination under the high power and oil immersion objectives. The various elements of bone marrow must be observed systematically viz: erythroid series, myeloid series, lymphocytes, monocytes, plasma cells, reticulum cells, megakaryocyte-platelet system and finally parasites. The maturity of cells and the erythroid myeloid ratio must be reported. Some typical reports are reproduced in the table on page 26.

INVESTIGATION OF HAEMOLYTIC ANAEMIAS

29. The clinical presentation of different haemolytic anaemias varies, hence the first step towards diagnosis is to suspect the presence of haemolytic process. Second step is to confirm its presence and the third or final step is to elucidate the aetiological factors involved in the mechanism.

The haemolytic anaemias may be acute or chronic. The acute condition usually has the picture of aches in limbs, rigor, fever, oliguria or anuria. Haemolysis is intravascular mostly, and this produces haemoglobinaemia, haemoglobinuria, methaemalbuminaemia and haemosiderinuria.

The chronic haemolytic disorder shows mild jaundice with enlarged spleen and liver. Haemolysis or destruction of red cells is extra-vascular ie in the reticuloendothelial cells. This produces hyper-bilirubinaemia with an increased icteric index and serum bilirubin. Faecal and urinary urobilinogen is increased.

Disease	Normal	Tropical macrocytic anaemia	Iron deficiency microcytic anaemia	Chronic myeloid leukaemia
Cellularity	Normal	Increased	Normal or increased	Increased
Predominant cell	Neutrophils & metamyelocytes	Megaloblasts and normoblasts in varying proportions	Normoblasts	Granulocytes
Granulocytes	All types but few early forms	A few giant metamyyelocytes and hypersegmented neutrophils	All types but few early forms. Cell size often small	All forms; many myelocytes. Basophils increased
Erythroblasts	Normoblasts and few erythroblasts	Megaloblasts and normoblasts in all stages	Normoblasts in all stages and with irregular scanty cytoplasm. Cell size small	Normoblasts in all sizes
Lymphocytes	Few, mature	Few, mature	Few, mature	Very few
Plasma cell	Few, mature	Increased often, mature types	Few, mature	Very few
Monocytes	Few, mature	Few, mature	Few, mature	Few (sometimes increased)
Megakaryocytes-platelet system	..	..	..	Megakaryocytes increased
Myeloid-erythroid ratio	2 : 1 to 8 : 1	1·5 : 1 to 7 : 1	1 : 1 to 4 : 1	3 : 1 to 7 : 1

To cope with this destruction of red cells, the marrow activity proliferates. Polychromasia, increase in reticulocytes and nucleated red cells in blood smears are indicative of marrow activity.

The essential feature of a haemolytic anaemia is the reduced life-span of red cells and this can be demonstrated by using Ashby's technique or better by Cr 51 method.

Detection of products of increased haemoglobin catabolism

30. *Serum bilirubin*: For technique refer to volume on Biochemistry page 70.

Significance: In haemolytic anaemias the value usually lies between 1—3 mgm/100 cc. Van den Bergh is mostly a direct negative. Values of serum bilirubin more than 5 mgm per cent with a direct positive Van den Bergh, indicate liver disease.

31. *Urobilinogen in faeces*

Principle: Stercobilin in faeces is reduced to urobilinogen and then treated with Ehrlich's reagent to give a pink colour which is compared in a colorimeter with a natural or artificial standard.

Technique:

A. Extraction:

(i) Weigh out in a (6″×1″) test tube 1.5 G of well mixed faeces.

(ii) Add 9 ml of water and emulsify with a glass rod.

(iii) Add 10 ml of ferrous sulphate solution. Mix well.

(iv) Add 10 ml of sodium hydroxide solution. Allow to stand for 2 hours with occasional stirring. Filter.

B. Test:

(i) To 2 ml of filtrate add 2 ml of Ehrilich's reagent. Mix. Allow to stand for 10 minutes.

(ii) Add 6 ml of sodium acetate.

C. Standard:

(i) To 1 ml of phenophthalein standard add 5 ml of sodium carbonate solution. Mix and add distilled water to make volume upto 100 ml. The colour of this is equal to that of 0.000387 mgm of urobilinogen per ml.

D. Blank:

(i) To 2 ml of faeces filtrate (from A) add 2 ml of hydrochloric acid solution and 6 ml of sodium acetate.

Compare test, standard and blank in a photo-electric comparator using a yellow-green filter (Ilford 625). Substract the blank reading from that of the test. If test solution is too coloured, then dilute suitably to get a colour comparable to that of the standard.

Calculation:

$$\frac{\text{Test—Blank}}{\text{Standard}} \times 3{\cdot}87 = \text{Urobilinogen in mgm per 100 G of faeces.}$$

Reagents:

(i) Ferrous sulphate: Dissolve 20 G of $Fe\,SO_4\,7\,H_2O$ in distilled water and make up to 100 ml.

(ii) 2.5 N—Sodium hydroxide: Dissolve 10G of NaOH in distilled water and make upto 100 ml.

(iii) Ehrlichs reagent: Mix 150 ml of concentrated hydrochloric acid with 100 ml of water. Dissolve in this mixture 0.7 G of P—dimethyl aminobenzaldehyde.

(iv) Sodium acetate: Make a saturated solution.

(v) 6N—Hydrochloric acid: Dilute 60 ml of concentrated acid with water to make 100 ml.

(vi) Standard phenolphthalein solution: Dissolve 50 mgm of phenolphthalein in 100 ml of ethanol.

(vii) Sodium carbonate: Dissolve 15 G of Na_2CO_3 in water and make volume to 100 ml.

Artificial standard (Watson et al)

This standard has a colour which more nearly matches the colour of urobilinogen.

Dissolve 5 mgm of "Pontacyl carmine 2B" and 95 mgm of "Pontacyl-violet 6 R 150 per cent" in 1 litre of 0.5 per cent (V/V) acetic acid. Now dilute 6 times with 0.5 per cent acetic acid. This is equivalent to 0.6 mgm of urobilinogen in 100 ml when treated with Ehrlich's reagent.

Significance:

The upper limit of normal per day is 280 mgm of urobilinogen. Abnormally increased values are a direct evidence of blood destruction. The test is valuable in haemolytic anaemias but the technique is not very accurate and four days collection of faeces is essential to make the specimen of faeces reasonably representative.

32. *Urobilin and Urobilinogen in urine*

(a) Qualitative test—technique given in Volume II (Biochemistry) Page 9.

(b) Semiquantitative measurement.

(i) Collect a 2 hour sample of urine between 2 PM and 4 PM with prior emptying of the bladder and taking a glass of water. Peak excretion of urobilinogen occurs usually during this part of the day.

(ii) Cool to room temperature.

(iii) Add 2.5 ml urine each to 2 colorimeter tubes marked *Blank* and *Test*.

(iv) To *Test* add 2.5 ml of Ehrlich's reagent, mix and add 5 ml of saturated sodium acetate solution.

(v) To *Blank* add 5 ml of sodium acetate solution, mix and then add 2.5 ml of Ehrlich's reagent. This reversal of reagent prevents colour development in the Blank.

(vi) Take reading for *Test* in photoelectric colorimeter at 565 mu using *Blank* for setting the centre of the colorimeter.

(vii) Calculate the concentration of urobilinogen in mgm per 100 ml urine by referring the Test reading to a calibration curve obtained by plotting the colorimeter readings of various dilutions of the artificial standard of Watson etal against the concentrations of urobilinogen in mgm per 100 ml.

(viii) Calculation of total urobilinogen in 2 hours urine—

$$\left.\begin{array}{l}\text{Concentration of final solution in mgm urobilinogen per 100 ml}\end{array}\right\} \times 4 \times \frac{\text{Urine Volume}}{100 \text{ ml}} = \text{Ehrlich units per 2 hours}$$

Significance:

Normal values are below one unit per two hours. Urobilin in urine is increased in hepatic dysfunction as well as increased red cell destruction; hence it is not a reliable index of haemolysis.

33. *Plasma haemoglobin*

(i) Using a syringe boiled in normal saline, make a 'clean' venepuncture and withdraw blood. Add 1.8 ml of blood to 0.2 ml of 3.8 per cent sodium citrate and mix.

(ii) Take three large test tubes and mark 'Test', 'Control' and 'Standard'. In "Test" place 0.1 ml of plasma. In the other two, place 0.1 ml of distilled water and 0.1 ml of haemoglobin solution of known haemoglobin content, respectively.

(iii) To each, add 2 ml of benzidine reagent and 1 ml of hydrogen peroxide solution and allow to stand for 1 hour at room temperature.

(iv) Add to each, 20 ml of 20 per cent (aqueous) V/V solution of glacial acetic acid. Colour develops in the tubes.

(v) Compare in a photoelectric colorimeter using a blue-green filter (Ilford 624). Calculate in mgm per 100 ml.

Reagents:

Benzidine solution: Dissolve 0.5 G of pure benzidine dihydrochloride (Merck) in 15 ml of hot distilled water and then add 25 ml of 95 per cent (v/v) ethanol and 10 ml of glacial acetic acid. Store in a dark bottle at 4°C. It keeps for several weeks.

Hydrogen peroxide: Make 0.6 per cent (v/v) solution of hydrogen peroxide.

Significance: Normal range is 1—4 mgm haemoglobin per 100 ml of plasma. Concentration is raised in haemolytic anaemias when haemolysis is predominantly intravascular. Marked haemoglobin anaemia is seen typically in Black water fever, paroxysmal cold and nocturnal haemoglobinurias. Mild increase is seen in sickle cell anaemia, thallassaemia major and acquired haemolytic anaemia.

34. *Haemosiderin in urine*

Method

(i) Centrifuge urine and remove the supernatant.

(ii) Add an equal volume of a freshly prepared mixture of equal volume of 2 per cent potassium ferrocyanide and 2 per cent hydrochloric acid and resuspend the deposit. Allow to stand at room temperature for 10 minutes.

(iii) Recentrifuge and examine a wet preparation of deposit under 4 mm objective of the microscope. Haemosiderin appears as blue staining granules 1—3 μ in size.

Significance:

It is a sign of chronic intravascular haemolysis. Persistent haemosiderinuria occurs in paroxysmal nocturnal haemoglobinuria. It is also seen in severe thallassaemia and acquired haemolytic anaemia. In fact it is seen in all cases where plasma continuously contains abnormal amounts of haemoglobin.

Methaemalbumin in plasma: For method refer to page 195 (biochemistry vol.) under spectroscopic examination of blood.

Erythrocyte fragility tests

35. *Osmotic fragility*

Method

(i) Make 1 per cent sodium chloride solution by diluting the stock solution with distilled water.

(ii) Prepare a range of dilutions of this saline solution as per chart on page 33, and add to each 0.05 ml of heparinized venous blood which has been shaken well in order to fully oxygenate it.

Tube No	1	2	3	4	5	6	7	8	9	10	11	12
ml of 1% Nacl ..	4·2	3·7	3·2	3·0	2·7	2·5	2·2	2·0	1·7	1·5	1·0	0·5
ml of dist water ..	0·8	1·3	1·8	2·0	2·3	2·5	2·8	3·0	3·3	3·5	4·0	4·5
Total volume	5·0	5·0	5·0	5·0	5·0	5·0	5·0	5·0	5·0	5·0	5·0	5·0
Concentration of Nacl ..	0·84	0·74	0·64	0·60	0·54	0·50	0·44	0·40	0·34	0·30	0·20	0·10
ml of blood	0·05	0·05	0·05	0·05	0·05	0·05	0·05	0·05	0·05	0·05	0·05	0·05

(iii) Allow to stand at room temperature (20° C) for 30 minutes.

(iv) Remix and centrifuge for 5 minutes at 2000 revolutions per minute. Separate supernatants into a separate set of tubes marked 1 to 12.

(v) Compare colour of all these with that of 100 per cent lysis tubes (tube No. 12 with 0.10 per cent salt concentration) in a photoelectric colorimeter. No. 1 tube supernatant serves as the *blank*.

Reagent

Stock sodium chloride solution:

(i) Weigh out the following:—

Sodium chloride	—180 G
Sodium hydrogen phosphate	—27.31 G
Sodium dihydrogen phosphate	—4.86 G

(ii) Dissolve these in distilled water and make to two litres. Store it in a well-stoppered bottle. It keeps well for months at 4°C.

Recording results of the test:

(i) A simple way is to note concentrations of salt solution in which initial (0—5 per cent), median (50 per cent) and maximal (100 per cent) lysis occur.

(ii) A better way is to draw a fragility curve by plotting on graph paper per cent lysis against concentration of salt solution. In normal persons this curve takes the form of a symmetrical sigmoid.

Normal values in osmotic fragility (at pH 7.4 and room temperature 20°C).

Initial lysis (0—5 per cent lysis)—0.50 per cent Nacl.

Median corpuscular fragility (50 per cent lysis)—0.40 per cent.

Maximal lysis (97—100 per cent)—0.30 per cent.

Significance—Osmotic fragility is a measure of spherocytosis. It is increased in hereditary spherocytosis and acquired and secondary haemolytic anaemias.

Osmotic fragility is decreased in simple iron deficiency anaemia, after splenectomy, in Thallassaemia, sickle-cell anaemia and in liver disease. Typical osmotic fragility curves in sickle cell anaemia, Thallassaemia, hereditary

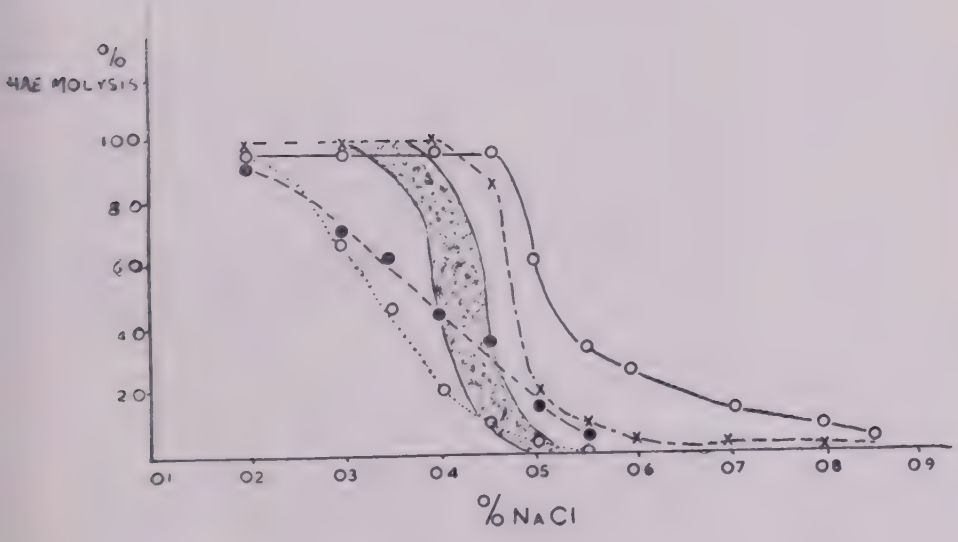


FIG 3 : OSMOTIC FRAGILITY CURVES

spherocytosis and idiopathic acquired haemolytic anaemia have been illustrated in figure 3. The normal range is represented by the shaded area.

36. *Osmotic fragility after incubation at 3*

Method

(i) Incubate in duplicate 2 ml volumes defibrinated blood in 5 ml screw cap sterile bottles at 37°C for 24 hours. (T bottles are used—in case one beco infected).

Carry out osmotic fragility as above putting concentrations of Nacl upto with an additional tube of 1.2 per Nacl (also) for providing "blank" colorimetric comparison.

Normal range (After incubation):

Initial haemolysis 0.60—0.75 per cent N Median haemolysis 0.50—0.55 per Nacl.

Maximal haemolysis 0.20—0.40 per cer

Significance:

Increased fragility is seen in hereditary s rocytic and some cases of congenital non-s rocytic haemolytic anaemia. It is valuabl spotting mild cases of hereditary spheroc sis.

37. *Autohaemolysis*

Method

(i) Incubate for 24 hours at 37°C, four 2 samples of defibrinated sterile blood 5 ml screw-capped sterile bottles.

(ii) Take out two bottles and mix blood. I bate the other two bottles for anothe hours.

(iii) Mix contents of first two bottles. De mine PCV and centrifuge remainde separate serum.

(iv) Compare in a photoelectric colorimeter the following:—

a. Test ... 1 in 50 dilution of serum in 0.04 per cent (v/v) ammonia.

b. Standard... 1 in 200 dilution of whole blood in 0.04 per cent ammonia.

(v) Similarly take readings for blood after 48 hours incubation and calculate percentage haemolysis separately.

Calculation:

$$\text{Percentage haemolysis} = R_s \times \frac{100 - PCV}{R_o \times 4}$$

R_o=Colorimetric reading of diluted whole blood.

P_s=Colorimetric reading of diluted serum

Normal range of autohaemolysis:

After 24 hours=0—0.5 per cent.

After 48 hours=0.4—3.5 per cent.

Significance:

Increased autohaemolysis is seen in hereditary spherocytosis, acquired haemolytic anaemia and spherocytosis, some cases of congenital non-spherocytic haemolytic anaemia. It may be found in paroxysmal nocturnal haemoglobinuria and in haemolytic anaemias due to chemicals like acetyl phenyl-hydrazine.

38. *Mechanical fragility*

Method

(i) Collect 10 ml of blood in a heparinized bottle.

(ii) Adjust packed cell volume to 45 per
by addition or withdrawal of plasma
necessary.

(iii) Deliver 2 ml of this blood into each of
5 ml tubes of size 8×1 cm.

(iv) Into each also place four glass beads
about 4 mm diameter. Close the tubes w
tight fitting rubber bungs.

(v) Rotate these tubes at 33 rpm for one ho

(vi) Pool the contents of both tubes and m
a 1 in 100 dilution of a portion of this
0.04 per cent ammonia. This makes
per cent lysis tube. Similarly make a 1
100 dilution in normal saline. This con
tutes the 'Test'.

(vii) A 'Blank' is made by having a 1 in
dilution of a pre-rotation sample of
blood in normal saline.

(viii) Determine the lysis in the 'Test' by co
paring its readings against the 100
cent lysis tubes, taken in a photoelect
colorimeter using a yellow-green fil
(Ilford 625).

Normal is 2—5 per cent haemolysis.

Significance:

The test measures the susceptibility
mechanical trauma. Spherocytes, sickl
cells and agglutinated cells are more su
ceptible to mechanical trauma, where
poikilocytes are not.

39. **Estimation of foetal haemoglobin**

Method:

A. Prepare a haemolysate of red cells take
from citrated, oxalated or heparinize
blood:—

(i) Wash red cells in normal saline thric

(ii) Mix 1 volume of packed cells with 1.5 volumes of distilled water and 0.5 volume of pure toluene. Shake for 5 minutes to lyse the cells completely.

(iii) Centrifuge the mixture at 3000 rpm for 20 minutes.

(iv) Discard the upper layer and the deposit. Take out the middle zone of clear red solution. This haemolysate keeps well at 44°C for many days. For use adjust haemoglobin to 10 G per cent in this solution.

B. *Alkali denaturation test*:

(i) Place 1.6 ml of N/12 Sodium hydroxide in a test tube and add 0.1 ml of haemolysate, starting the stop watch at the same time and rinsing pipette several times in the alkali. Mix well.

(ii) After exactly 1 minute add 3.4 ml of precipitating solution. Mix thoroughly. A brownish precipitate forms.

(iii) Filter through Whatman No. 42 filter paper.

(iv) Take a reading of the colour of the filtrate in a Beckman spectrophotometer at 540 mu. This is reading 'A'.

(v) Add 0.02 ml of haemolysate to 4 ml of distilled water and take a reading of its colour in spectrophotometer. This is reading 'B'.

Calculation:

$$\frac{A}{4 \times B} \times 100 = \% \text{ Haemoglobin F}$$

Reagents:

(i) Stock sodium hydroxide solution: Pre
N/4 NaoH and keep it in tightly stopp
paraffinized bottle. Dilute one part of
with two parts of distilled water to
N/12 NaoH.

(ii) Precipitating solution: Mix the foll
ing:—

Saturated amm sulphate	—	500
Distilled water	—	500
11 N Hydrochloric acid.	—	2.5

Normal range:

As the technique is insensitive to less tha
per cent HbF, figures below 2 per cent
considered normal.

Significance:

It is present in new born infants (55—85
cent) but it usually falls to 1 per cent after
age of 6 months to one year.

Increased amounts are seen in homozyg
thallassaemia and other homozygous haer
globinopathies.

40. **Paper electrophoresis of haemoglobins:**

The apparatus consists of two buffer chamb
made of perspex and each divided into 2 s
tions connected by glass agar bridges or
small holes near the bottom of the partiti
The outer section contains a platinum el
trode. A power pack provides a stabiliz
direct current of approximately 100—400 vol
Veronal buffer: pH 8.6 and ionic streng
0.05.

Barbitone sodium	..	5G
Hydrated sodium acetate	..	3·33 G
0·1 N Sulphuric acid	..	34·2 ml
Hydrated copper sulphate	..	1 mg
Water to	.. ..	1 litre

Method:

(i) Place buffer in the buffer chambers so that the fluid is at the same level in both chambers.

(ii) Cut electrophoresis Whatman No. 3 MM paper strips of size $18'' \times 4\frac{1}{2}''$.

(iii) Draw a pencil line in its middle. Soak the paper in buffer and blot it dry. Apply the haemolysate on the pencil mark in the form of a drop or a streak.

(iv) Place the paper between two glass plates of size $12'' \times 4\frac{3}{4}'' \times \frac{1}{4}''$ so that about 3″ of paper hangs free on both ends of the plates. Seal the edges of glass plates with vaseline grease and apply clamps on sides to ensure equal pressure on glass plates.

(v) Now keep the plates between the two chambers so that their ends rest on the inner margin of the chambers and the paper dips into buffer in the inner sections.

(vi) Buffer will soak into the paper. Let it stabilize for $\frac{1}{2}$ hour.

(vii) Connect terminals with power pack and switch on the current. Voltage may be between 100 to 400 volts. Allow current to pass for 6 hours. Most of the haemoglobins separate by this procedure and are visible to the eye.

(viii) Separate the terminals, remove the paper and dry it in a hot air oven at 110°C for 15 minutes.

(ix) Haemoglobins are visible but if they are to be permanently recorded, they can be stained with the following solution:—

Bromophenol blue ... 0.1 G
$Hg\ cl_2$ ... ... 10 G
95 per cent alcohol upto 100 ml (finel vol).

Put dry paper in stain for 10 minutes. Th
wash with tap water to remove the excess d
Exact identification of haemoglobins is achi
ed by putting known specimens of haemog
bins also on the electrophoresis paper a
running them in parallel with the unknow
sample. These known samples serve as '*ma*
kers'.

41. **Demonstration of sickling: (26)**

Method

(i) Add 0.05 ml of reducing solution (2 p
cent sodium bi-sulphite) to 0.01 ml
blood on a slide and cover it with a cove
slip immediately.

(ii) Seal the moist preparation with a pet
leum jelly and paraffin mixture.

(iii) Incubate at 37°C for 1 hour. Examine w
the low and high dry objectives of t
microscope. If negative, incubate for fu
ther 24 hours.

Significance:

Sickling is marked in sickle cell anaemia.
the sickling trait the phenomenon is less mar
ed and "holly-leaf" forms are prevalent.

SEROLOGICAL INVESTIGATION OF ACQUIR
HAEMOLYTIC ANAEMIAS

42. In majority of cases of acquired haemoly
anaemia the increased destruction of red cells is d
to the action of auto-antibodies absorbed on the su
face of red cells or free in the serum. These antibod
do not present a consistent pattern and differ fro
case to case in their temperature requirement, chem
cal nature, specificity and reactions in the test tub
Antibodies may be warm (act well at 37°C) or of co
type (act well below 37°C). Further, mo

these are incomplete antibodies ie these sensitize ed cells to the action of antiglobulin serum but do not roduce agglutination in a saline medium.

43. **Collection, storage and transport of Samples**

(i) Serum is separated from blood clotted at 37°C. It is best stored at −20°C.

(ii) Red cell suspension: Red cells are washed thrice in normal saline and then suspension made in normal saline in the required concentration. Red cells can be preserved at 4°C for 2-3 weeks, if collected in acid-citrate dextrose (ACD) anticoagulant.

(iii) If the samples are to be sent by post to a distant lab, send separately serum (separated at 37°C) and blood collected in bottle containing acid citrate dextrose as anticoagulant.

44. **Combs' Test**

This is a method which detects incomplete antibodies absorbed on to red cells, which do not bring about agglutination in saline dilutions of serum. These cells, if washed thrice in normal saline in order to remove serum or plasma and the anti-human globulin rabbit serum added, undergo agglutination.

Direct qualitative test:

(i) Wash patient's red cells thrice in warm normal saline (at 37°C). Prepare 10 per cent suspension in normal saline.

(ii) Mix one drop of this with one drop of potent anti-globulin serum diluted to the point of its maximum avidity, on a translucent tile.

(iii) Rock the suspension gently from time to time. At the end of 5 minutes look for agglutination and if present record its

strength as ±, +, ++, +++, ++++. The last denotes the strongest reaction which is complete within a few seconds.

(iv) Controls: It is good to do the test on normal red cells (specificity control) and sensitized red cells (sensitivity control). Red cells can be sensitized with warm or cold antibodies:—

(a) Warm antibody-sensitized cells are made by suspending 1 volume of Group-O (D-positive) cells in 9 volumes of incomplete anti-D serum. Keep suspension at 37°C for 2 hours. After 2 hours centrifuge and wash the cells in normal saline.

(b) Cold antibody sensitized cells are prepared by suspending 1 volume of 50 per cent Group-O cell suspension in 9 volumes of fresh normal serum (known to contain cold antibody of incomplete type). Keep at 0°C for 2 hours. Centrifuge and wash thrice in saline.

Direct quantitative test:

(i) Prepare four-fold dilutions of antiglobulin serum in a set of tubes (range ¼ to 1/4096).

(ii) Place one drop from each tube on a tile and mix 1 drop of 10 per cent suspension of patient's cells. One drop of saline with one drop of red cell suspension, serves as a control. Rock the tile to mix the suspensions.

Significance:

A positive test shows the presence of auto-anti-bodies but is not always diagnostic of acquired haemolytic anaemia as false positives

are often recorded in variety of diseases eg. rheumatoid arthritis, lupus erythematosis, leukaemia, apiastic anaemia, sarcoidosis. However, these are usually weak reactions and maximal in low dilution of serum.

45. **Indirect Comb's Test**

This test is positive when warm auto-antibodies are present free in the serum. This, however, happens only in serious cases of acquired haemolytic anaemia. In mild cases these antibodies cannot be demonstrated by this test unless enzyme treated red cells are used.

A. *Method*

(i) Add 2 drops of 2 per cent suspension of washed red cells of normal groups O (CDe/cDE) in a 50 mm×7 mm tube containing 2 drops of patient's serum.

(ii) Another tube contains 2 drops of normal saline to which 2 drops of cell suspension are added. This serves as a control.

(iii) Incubate these tubes at 37°C for half an hour.

(iv) Then withdraw red cells from the deposit in the tube and wash these thrice in three changes of saline.

(v) Now proceed as for the direct test using these cells and antiglobulin serum.

B. *Method using enzyme treated cells*

(i) Prepare doubling dilutions of serum ranging from 1/1 to 1/256 in a set of tubes placed in water bath at 37°C.

(ii) Add equal volume of 1 per cent suspension of enzyme-treated red cells to each tube and mix.

(iii) After 2 hours take readings for agglutination observing the deposit of cells at the bottom of the tubes with a concave mirror.

Reagent:

Preparation of trypsinized red cells.

(i) Stock trypsin solution. Make a 1 per cent solution of crystalline trypsin in 0.05 N HCl. This keeps well at 4°C for a week.

(ii) For use make a 0.1 per cent solution by diluting one part of stock solution in nine parts of phosphate buffer of pH 7.7 (made by mixing 90.5 parts of 1.63 per cent Na_2HPO_4 anhydrous and 9.5 parts of 2.34 per cent NaH_2PO_4 $2H_2O$).

(iii) To 1 ml of 0.1 per cent trypsin solution add 0.2 ml of packed washed normal 'O' groups CDe/cDE cells. Incubate for 1 hour at 37°C. Wash these red cells twice in normal saline.

46. **Demonstration of cold agglutinins**

(i) Make doubling dilutions of serum ranging from 1/1 to 1/2048. Add an equal volume of 1 per cent normal group 'O' red cells to each tube and mix. Let stand at 20°C (room temperature). Read agglutination microscopically using a concave mirror.

(ii) Remix suspensions and place the tubes in a refrigerator at 4°C for 2 hours. Read agglutination again, using a concave mirror. Record the titre of agglutination.

Significance:

Titres of 40 and higher have been recorded in 68 per cent cases of primary atypical pneumonia and in some cases of acquired haemolytic anaemia. Rarely the titre is high in other conditions eg cirrhosis of liver, trypanosomiasis.

Demonstration of Haemolysins

47. *Simple presumptive test*

(i) Place 2 ml of defibrinated blood in a test tube. Incubate at 37°C for 2 hours.

(ii) Centrifuge. Presence of haemoglobin in supernatant suggests presence of warm haemolysin.

(iii) Place 2 ml of defibrinated blood in another tube and keep at 0°C for 20 minutes. Look for agglutination in the deposit with a concave mirror. Its presence suggests cold agglutinins.

(iv) Incubate this tube at 37°C for one hour. Presence of haemoglobin in supernatant suggests a cold haemolysin. If this is positive Donath-Landsteiner test should be done.

48. *Donath-Landsteiner test*

(i) Collect 2 ml of patient's blood in a test tube and allow it to clot.

(ii) Similarly collect 2 ml of a normal person's blood and allow it to clot. This is a normal control.

(iii) Place both test tubes in ice water for 10 minutes.

(iv) Warm in a water bath at 37°C for 30 minutes.

(v) There will be no haemolysis in the control. Haemolysis in patient's serum will show a positive DL test.

Significance:

The test is positive in paroxysmal cold haemoglobinuria.

49. *Ham's Test*

(i) Obtain serum from patients' defibrinated blood.

(ii) To 0.5 ml of this, add 0.05 ml of N/5 Hcl and mix carefully.

(iii) Add 0.05 ml of 50 per cent suspension of washed red cells obtained from the patient. Incubate for 1 hour. Centrifuge. Definite lysis in supernatant indicates a positive test.

(iv) Put up a *control* using the patient's serum and normal red cells similar to above. It should not show any haemolysis or maximally a trace of it.

Significance:

The test is diagnostic of Paroxysmal nocturnal haemoglobinuria. The red cells in this condition are lysed by normal constituents of serum at an acid pH(6.5—7.0).

50. **Demonstration of Heinz bodies**

(i) Examine an unstained blood smear under the microscope with reduced illumination. Heinz bodies appear as refractile particles 1—3 μ in size lying close to the cell membrane.

(ii) For better results mix equal volumes of blood and 0.5 per cent methyl violet in normal saline. Allow to stand for 10 minutes at room temperature. Make films. Heinz bodies are seen as intense purple particles.

Significance:

Presence of Heinz bodies indicate usually chemical poisoning of exogenous origin. A number of aromatic amino and nitro-compounds as well as inorganic oxidizing agents

can lead to production of these inclusions in the red cell. Typical examples are phenyl hydrazine and potassium chlorate.

ABORATORY INVESTIGATION OF HAEMORRHAGIC AND PURPURIC DISORDERS

51. Purpuric and haemorrhagic conditions can be ue to a defect in the capillaries, defective platelet unction or a deranged coagulation mechanism. The rio of, Hess's capillary resistance test, bleeding time nd clotting time, roughly enable a condition under ıvestigation to be put in one of the above three aetiogical groups. The tests are, however, crude and their ormal range wide so that mild abnormalities give esults which fall into the normal range. The tests ıould be repeated often and several observations ıade to confirm abnormal findings.

If Hess's capillary resistance test is positive, it ıould be repeated as a quantitative test. Prolonged leeding time indicates not only a reduced quantity of latelets but this is also a measure of vascular conractility. As this test is not very sensitive, it must e supplemented with total platelet count and clot etraction. The latter is a measure of platelets and to ome extent of fibrinogen.

A prolonged clotting time may be due either to a eficiency of clotting factors or to the presence of cirulating anti-coagulants. To resolve this, one should o three tests viz. thromboplastin generation test, rothrombin time and fibrinogen estimation. These ree detect the deficiency of the various clotting facors.

52. **Hess's capillary resistance test**

(i) Tie the pneumatic cuff of the Sphygmomanometer on the arm and raise the pressure to a point midway between systolic and diastolic pressures.

(ii) Keep it for 5 minutes only. If petechiae appear on the limb below the place where the cuff was tied, the test is said to be positive.

Quantitative test

(i) Mark a circle 6 cm in diameter in antecubital fossa and note if any petechiae or blemishes are already there in the circle. Mark these with ink.

(ii) Tie a pneumatic cuff on the arm 1" above the circle and raise pressure to 50 mm Hg and keep it for 15 minutes only. Remove the cuff.

(iii) Count the number of petechiae in the circle by illuminating the arm with a 30 watt bulb at two feet from the arm.

Normal is 0—8 petechiae.

53. Bleeding time

A. *Duke's method*

Make a moderately deep cut (3 mm) in the pad of finger by sharp blood lancet so that blood oozes without exerting any pressure on the finger. Start a stop watch at the same time. Touch the drop every half minute with the edge of a filter paper taking care that it does not touch the edges of the wound. Note the time when bleeding spontaneously ceases.

Normal bleeding time is 1—5 minutes.

B. *Ivy's method*:

Tie a pneumatic cuff on the arm and raise pressure to 40 mm of mercury. Select an area on forearm free of visible veins, clean with alcohol and puncture with a blade to a depth of 3 mm. Blot the blood every half a minute and note the time when oozing stops at the site of puncture.

The normal bleeding time with this method is 2—6 minutes. This test is more sensitive than Duke's, as it is perfomed on a less vascular area and variations caused by alterations in capillary tonus is controlled.

54. **Coagulation time**

A. *Lee & White's method*

(i) Draw venous blood in a dry syringe, detach the needle and pour 1 ml of blood into each of two test tubes; keep in an incubator at 37°C. Start a stop watch as soon as venepuncture is made and blood enters the syringe.

(ii) Examine one of the tubes every ½ minute and look for clotting by tilting the tube. Note the time when the tube can be tilted to an angle of 90° without spilling.

(iii) Now examine the other tube and note time of clotting. Mean of the readings from the two tubes gives the coagulation time.

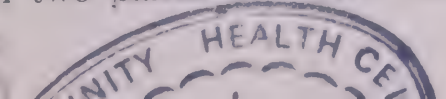
Normal coagulation time with this method is 5—11 minutes.

B. *Capillary tube method*

(i) Prick a cleansed finger and start stop watch.

(ii) Fill blood into capillary glass tubes by touching these to the edge of the drop of blood. Blood automatically flows into a capillary tube kept at an incline downwards.

(iii) Keep these tubes in the incubator at 37°C or between two palms of warm hands.



(iv) After a lapse of 3 minutes, at every minute interval, break small bits of capillary tube and note the time when a fibri thread appears between the broken end This indicates the coagulation time. Nor mal clotting time with this method is 4— minutes. This test is insensitive but highl convenient to perform. It fails to detec mild cases of clotting disorders.

Significance of clotting time

The tests, even if carefully done, are inherently insensitive and non-specific. Clotting is prolonged in haemophilia, Christamas disease conditions due to circulating anti-coagulant prothrombin deficiency and during hepari administration. It may or may not be increased with tromexan and dicoumarol thereapy and in fibrinogenopenia.

55. **Clot retraction**

In this test a volume of serum produced from blood clotted at 37°C is recorded and expressed as a percentage of the volume of the whol blood.

Method

(i) Withdraw venous blood into a clean dr syringe and deliver 5 ml into a graduate 10 ml centrifuge tube.

(ii) A wire with a loop at the lower end i inserted into the tube and is held verti cally by means of a cork.

(iii) Incubate the tube at 37°C (continue incu bation for an hour after clotting ha occurred). Allow to stand at 37°C for on hour after it has firmly clotted.

(iv) The clot adheres to the loop and retract around it. Carefully remove the wire wit the clot attached and hold the clot to th edge of the tube to drain for one minute

(v) Read the volume of serum and cells collectively from the graduations on the tube. Express this as a percentage of 5 ml.

Normal limits: It varies between 45—65 per cent with a mean of 55 per cent.

Significance:

Clot retraction is a measure of platelet function.

Clot retraction is poor in thrombocytopoenias.

56. **Prothrombin time by Quicks' one stage test**

The principle of the test is to mix optimal quantities of tissue thromboplastin, calcium and autogenous fibrinogen. The clotting time of this mixture is inversely proportional to the amount of prothrombin present and this is a valuable test in the control of anti-coagulant therapy. The test is affected by a deficiency of factor VII and V, especially the former. Anti-coagulant therapy reduces these factors more and hence "prothrombin time" is a measure of prothrombin and factors VII & V.

Method

(a) *Collection of plasma*:

(i) Withdraw blood by a clean venepuncture and pour 4.5 ml into a special anticoagulant tube containing 0.5 ml of 3.8 per cent sod citrate. (An alternative anti-coagulant is 0.5 ml of M/10 potassium oxalate solution made by dissolving 1.84 Gm of anhydrous potassium oxalate in 100 ml of distilled water). Mix well. Centrifuge and separate plasma. This keeps well at 4°C for 4—6 hours.

(ii) Collect normal blood in the same manner as above and separate plasma. This serves as a control.

(b) *Preparation of reagents*:

Stock thromboplastin powder. Remo human brain within 24 hours of dea Take a coronal slice 5 cm thick from t cerebral hemisphere. Cut it into small b and grind them in a mortar with 250 of acetone. After 5 minutes decant t acetone and grind again with a fresh 2 ml of acetone. Carry out this procedu 5 times. Express the mass between lar sheets of blotting paper and then spread out on a fresh sheet to dry. Grind it reduce it to the consistency of sand a sieve it through a wire gauze. Grind t big heavy particles again. Now keep dessicator for 24 hours and then seal 0 gm quantity in ampules. Store these 4°C. It keeps well for 3 months.

Working thromboplastin solution: S pend the contents of one ampule in 2.5 of normal saline. Shake well and use t supernatant for the test. This soluti keeps active for 48 hours at 4°C.

M/40 Calcium chloride solution: Dissol 2.775 gm of pure anhydrous (analar) c cium chloride in 1000 ml of water.

(c) *Test*:

(i) Place tubes containing thromboplas solution, calcium chloride solution a plasmas under test, in a water bath 37°C till they reach this temperature.

(ii) In a Kahn tube kept at 37°C in the wa bath, place 0.1 ml of plasma.

(iii) Add 0.1 ml of working thromboplas solution and shake well to mix. Keep one minute.

(iv) Add 0.1 ml of calcium chloride solution and start a stop watch. Shake it gently to mix. After 4-5 seconds lift it out of the water bath and tilt it horizontally so that the contents run back and forth at eye level. Appearance of fibrin clot denotes the end point. Stop the watch and note time in seconds. Mean of three such readings gives the correct time.

(v) Repeat the test with normal control plasma.

(d) *Results*:

(i) The clotting time of normal plasma falls between 11—17 seconds differing with each batch of thromboplastin. The prothrombin time of the unknown is reported along with the time of normal plasma.

(ii) Another way of reporting is to express this as prothrombin index by the formula

$$\frac{\text{Normal "Prothrombin Time"} \times 100}{\text{Patient's "Prothrombin Time"}}$$

(iii) It is often expressed as a percentage by referring 'Prothrombin time' in seconds of test sample on calibration curve drawn by plotting on graph paper clotting time in seconds against various concentrations of normal plasma as illustrated below:—

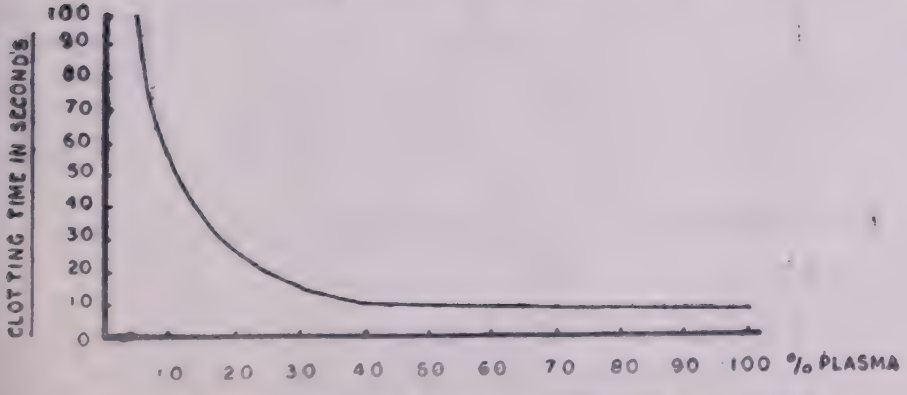


FIG 4—*Prothrombin time calibration curve.*

(e) *Significance*: The test is used as a contro in therapy with dicoumarol, tromexan an dindevan. It is also of value in the diagno sis of coagulation disorders. A norma prothrombin time rules out major defi ciencies of prothrombin and factors VII & V fibrinogen.

(f) *Fallacies*:

(i) Prothrombin time is prolonged if fibrino gen content of plasma falls below 30 per cent of normal.

(ii) Presence of excess of anti thrombins like heparin lengthens the apparent prothrom bin time.

57. Qualitative test for deficiency of factor V

Method

(i) Add 0.1 ml of alumina-absorbed normal plasma to 0.9 ml of patient's plasma.

(ii) Conduct "Prothrombin time" test on this mixture as well as on patient's plasma.

(iii) For preparation of alumina-absorbed nor mal plasma refer to para 59(c).

Results

The prothrombin time of patient's plasma will be appreciably longer than the prothrombin time of the mixture, if patient has a deficiency of factor V. Alumina-absorbed normal plasma is rich in factor V but poor in factor VII and prothrombin.

58. Qualitative test for deficiency of Factor VII

Method

(i) Collect 3 ml of blood in a glass tube con taining 3 glass beads and shake during

clotting. This ensures conversion of most of prothrombin to thrombin.

(ii) Let stand at 37°C for one hour and then separate serum by centrifuging.

(iii) Add 0.1 ml of this serum to 0.9 ml of patient's plasma.

(iv) Conduct "prothrombin time" tests on this mixture as well as on patient's plasma separately.

Result

If prothrombin time of mixture is significantly less than the time of patient's plasma, it indicates deficiency of factor VII in the patient. Serum is rich in factor VII but contains negligible quantities of prothrombin and factor V.

59. **Thromboplastin generation test**

Principle:

Five components viz. platelets, antihaemophilic globulin, christmas factor, factor V and factor VII act in the presence of calcium chloride and produce thromboplastin. Advantage is taken of the knowledge that normal alumina-absorbed plasma contains factor V and antihaemophilic globulin only, whereas normal serum contains factor VII and Christmas factor but not the antihaemophilic globulin and factor V. These reagents ie platelet suspension, alumina-absorbed plasma, and serum are obtained from the patient as well as from a normal individual. Various combinations of these are made and the amount of thromboplastin generated is measured by finding the clotting time of a mixture of this thromboplastin and substrate plasma. The test is interpreted in combination with the "prothrombin time" test and enables the detection of a defect of any of the above listed five components.

Specimen, reagents and apparatus:

(a) *Siliconed glassware.*

(i) Clean new glassware scrupulously wit soap and then dichromate mixture. Dry i

(ii) Pour silicone fluid M441 (ICI) into glas tubes, centrifuge tubes, flasks etc an draw it into syringes, needles, pasteu pipettes and then let these drain back int the bottle of silicone.

(iii) Then rinse these articles in distille water. Rinse once with very dilute am monia and then copiously with distille water. Dry in hot air.

(iv) Repeat the whole treatment three time and then store in dust proof containers.

(v) After use wash the apparatus, dry and recoat.

(b) *Platelet suspensions and substrate plasma.*

1. Collect 20 ml of normal whole blood using a siliconed syringe and needle and pour into two 10 ml siliconed graduated centrifuge tubes containing 1 ml each of 3.8 per cent sodium citrate and mix.

2. Centrifuge at 1500 rpm for 10 minutes and separate platelet rich plasma in siliconed tubes.

3. Centrifuge at 3000 rpm for 15 minutes and separate supernatant plasma. This is substrate plasma.

4. Wash the deposit of platelets twice with normal saline. Use a wooden stick for fragmenting the mass of platelets and centrifuge for redepositing.

5. Suspend the deposit in 3.5 ml of normal saline. This will keep for several days if stored at —20°C.

(c) *Alumina-absorbed plasma*: (For preparation of alumina refer to para 69).

1. Collect blood with a siliconed syringe and add it to a one-tenth volume of 3.8 per cent sodium citrate in a siliconed tube. Separate plasma by centrifuging at 3000 rpm for 15 minutes.

2. Add 0.1 ml of alumina to 1 ml of plasma. Mix and allow to stand at 37°C for five minutes. Centrifuge and preserve the supernatant. "Prothrombin time" of this plasma should be 1—4 minutes.

3. Dilute this plasma 1 in 5 with veronal buffer at pH 7.35 (refer to para 70).

(d) *Serum*: Pour 3 ml of blood into a tube with 3 glass beads, stopper it and invert repeatedly till blood clots. Allow it to stand at 37°C for 2 hours. Centrifuge and separate the serum. Dilute 1 in 10 with normal saline. This remains active for many days if stored at 4°C.

Method:

1. Place 0.1 ml of substrate plasma into each of 6 Kahn tubes placed at 37°C in a water bath.

2. In another tube at 37°C place 0.3 ml of alumina-absorbed plasma, 0.3 ml of platelet suspension and 0.3 ml of serum. Mix well.

3. Add 0.3 ml of M/40 calcium chloride a start stop watch. At intervals of 1 min withdraw from this mixture 0.1 ml us a graduated Pasteur pipette and draw ml of M/40 Cacl, into a second tube. D charge the contents of both pipettes sim taneously into one of the tubes containi substrate plasma and record its clotti time. Carry out the experiment upto to of 6 minutes.

Results:

Clotting times of substrate afford measure of thromboplastin in the mixtu and thromboplastin concentration is calc lated by using a thromboplastin diluti curve. This curve is prepared by maki potent thromboplastin by mixing t above reagents, derived from a norm individual. After 5 minutes' incubati place it in ice water. Then make dilutio of this ranging from 5 per cent to 95 p cent with normal saline. Determine t clotting times of substrate plasma wi these dilutions of thromboplastin as de cribed above. Draw a curve relating clo ting time and thromboplastin concentr tion; 100 per cent thromboplastin shou give a clotting time of about 10 seconds.

Significance:

The test is helpful in differentiating ha mophilia, Christmas disease and circula ing anticoagulants from one anothe Typical findings of this test in the variou conditions are shown in the table belov Prothrombin time findings help in th differentiation of factors V and VII.

Source of { Pl telets / Al. Plasma / serum	Mixture of Patient / Normal / Normal	of reagents Normal / Patient / Normal	Normal / Normal / Patient	Prothrombin time
Haemophilia	N	Abnormal	N	N
Christmas disease	N	N	Abnormal	N
Thromboplastin inhibitor	N	Abnormal	Abnormal	N
Thromboasthenia	Abnormal	N	N	N
Factor V deficiency	N	Abnormal	N	Prolonged
Factor VII deficiency	N	N	Abnormal	Prolonged

Table showing correlation of thromboplastin generation test and prothrombin time in various clotting disorders.

N *denotes normal.*

60. Demonstration of circulating anticoagulants

Spontaneously occurring anticoagulants are sometimes responsible for producing haemophilia like conditions. Usually these are thromboplastin inhibitors but rarely these may be antithrombins (heparin or heparin-like).

(a) Thromboplastin inhibitors can be detected by thromboplastin generation test. A simple way of finding the presence of these inhibitors is to mix 1 volume of patient's blood with 9 volumes of normal blood and note the clotting time of the mixture which will be significantly larger than that of normal blood alone.

(b) Detection of heparin-like activity.

1. Prepare two such dilutions of thro bin that they will clot normal plas in 8 and 20 seconds respectively.
2. Add 0.1 ml of each to 0.1 ml of norm plasma samples diluted with 0.1 ml saline. Note the clotting times.
3. Similarly note the clotting times usi patient's plasma instead of norm plasma.
4. If the clotting time of patient's plasn is more than that of normal plasm add 0.1 ml of 0.05 per cent solution toluidine blue to patient's plasn instead of adding saline and repe the experiment. If the clotting time this returns to normal, it can be pr sumed that heparin like activity present.

Estimation of fibrinogen: For metho refer to Biochemistry Vol. page 126.

MISCELLANEOUS TESTS

61. Demonstration of LE Cells

The LE cell phenomenon is seen almost exclusive ly in peripheral blood and marrow preparations o patients suffering from disseminated lupus erythema tosis and it depends upon the presence of a factor, a Y globin in their plasma. This factor lyses the nuclei o neutrophils which are subsequently phagocytosed b other neutrophils to produce the LE cell. This cel when stained by Ramonowsky stains appears as a neu trophil containing a larger spherical, pale purple opaque homogeneous mass in the cytoplasm with the nucleus of the ingesting leucocyte displaced to one side The LE cell may rarely be a monocyte or an eosino phil.

ethod:

1. Collect 10 ml of blood in a 100 ml flask containing 10—15 glass beads. Keep on shaking for about 10 minutes to completely defibrinate it.
2. Incubate at 37°C for 2 hours.
3. Pour this into two Kahn tubes and centrifuge at 3000 r.p.m. for 5 minutes. Transfer the buffy coat with a little of overlying serum to a Wintrobe tube and centrifuge for 5 minutes at 3000 r.p.m.
4. Make films from the buffy coat. Dry quickly and fix in methanol and stain with a Ramanowsky stain.

An alternative method is to separate patient's rum and mix with this a suspension of washed leucotes derived from a Group O individual. This is incuated at 37°C for 2 hours and then smears made.

62. **Erythrocyte sedimentation rate (ESR)**

Westergren's method:

1. Draw venous blood into a syringe and transfer exactly 2 ml to a bottle containing 0.5 ml of 3.8 per cent sodium citrate solution. Mix well.
2. Suck up blood into a Westergren tube to the mark 200 mm. Place the tube vertically in the Westergren stand.
3. After exactly 1 hour read the height of clear plasma above the upper limit of sedimenting red cells. This height in mm is the ESR per first hour.

Normal range:

Men	3—5 mm in one hour
Women	4—7 mm in one hour

The Wintrobe and Landsberg method is also sometir used but is not described here and is not recommend

Significance:

The test is widely used in clinical medicine bu empirical. It is increased in most infections, toxaem injections of foreign proteins, conditions accompan by hyperglobulinaemia and hypercholesterolaem The rate is decreased in polycythaemia, congest heart failure and sometimes in iron deficiency anaem

An increased ESR is always abnormal where normal ESR indicates health or mild disease. Thus t test helps to differentiate organic disease from fu tional. A rising sedimentation rate suggests a progr sive disease.

63. **Peroxidase Staining**

The cytoplasm of granulocytes and monocyt contains peroxidases which enable hydrogen peroxi to oxidise benzidine. Oxidised benzidine forms a bl compound with sodium nitroprusside.

Technique of staining (Washburn's method):

1. Add 10 drops of solution I to fresh thin dr blood films. Allow this to act for 1½ minute
2. Add 5 drops of solution II. Allow to act fo 4½ minutes. Dry it in air.
3. Stain with Leishman's stain in the usual man ner using a buffer.

Reagents:

Solution I:

Dissolve 0.3 Gm of benzidine in 99 ml of etha nol and add 1 ml of saturated sodium nitro prusside solution. This solution keeps for abou 6 months.

Solution II:

Mix 6 drops of hydrogen peroxide (20 Vol) in 25 ml of water. This should be prepared fresh just before use.

Significance

Granulocytes and myelocytes contain plenty of oxidase granules. Myeloblasts may contain a few granules. Monocytes and promonocytes are often faintly oxidase-positive.

APPENDIX

64. Preparation of syringes

All-glass syringes of 10 or 20 cc capacity and 19 20 SWG needles are placed in wide, long, test tub containing a thick pledget of cotton at the base so th the syringe and the needles separately rest on t cotton. The mouth of the tube is plugged with cott and covered with paper. These are sterilized in a h air oven at 160°C for one hour. Application of vaselir on the pistons of syringes before sterilization is r commended as this facilitates easy movement of th piston as well as delays coagulation of blood.

Use of syringes with iron pistons is considered ir ferior. Unless these are sterilized in an autoclave, som amount of fluid is always left in them and this dilute the sample of blood. (For prevention of haemolysi they should be sterilized by boiling in normal salin and never in distilled water).

65. Preparation of Wintrobe's Anticoagulant Bottles

Clean small bottles (*e.g.* Penicillin bottles from the wards) are sterilized in a hot air oven and 0.2 ml of a solution containing 0.8% potassium oxalate and 1.2% ammonium oxalate is placed in each. They are dried by placing in an incubator for a few hours. Each is now covered with a clean rubber bung to prevent absorption of plasma which would happen with cotton wool. This bottle is suitable for 2 ml of blood and provides anti-coagulant salt mixture at a concentration of 2 mg per ml of blood.

66. Cleaning of Glassware

Dichromate cleaning fluid—Dissolve 20 G of Potassium dichromate in 100 ml of distilled water and then add 900 ml of concentrated sulphuric acid.

New Slides:

Place these in dichromate cleaning fluid for 48 ours, then wash in running tap water. Rinse in dis- led water and store in 95% ethyl alcohol. Wipe and y with clean muslin cloth before use.

Used Dirty Slides:

After use, slides are thrown into 2% chlorosol. ash in tap water and boil in washing soda solution r 20 minutes. Wash in tap water and rinse with 5% ydrochloric acid. Wash in tap water and then distilled ater. Wipe and dry with clean muslin.

67. **Buffer Solutions**

A buffer of any pH can be made by mixing in fferent proportions the following two stock solu- ons:

A. M/15 Potassium dihydrogen phosphate solution: Dissolve 9.08 G of analar anhydrous KH_2PO_4 in water and make up to 1 litre.

B. M/15 Disodium hydrogen phosphate solution— Dissolve 9.47 G of analar anhydrous Na_2HPO_4 in water to make 1 litre.

pH	M/15 KH_2PO_4 ml	M/15 Na_2HPO_4 ml
6·5	6·82	3·18
6·6	6·30	3·70
6·7	5·66	4·34
6·8	5·08	4·92
6·9	4·48	5·52
7·0	3·89	6·11
7·1	3·34	6·66
7·2	2·80	7·20
7·3	2·32	7·68
7·4	1·92	8·08
7·5	1·59	8·41

68. **Preparation of solutions of Ramonowsl stains**

Leishman stain—Dissolve 0.15 G of powdere stain in 100 ml of methyl alcohol (acetone free) b grinding the stain with successive small quantities alcohol till it is all dissolved. Keeping in full ligh upto six months improves the staining property.

Giemsa Stain

Transfer I G of powdered stain to a conical flask Add 66 ml of glycerol and heat at 56°C for 1½— hours. Add 66 ml of methyl alcohol (acetone free and allow to stand for 7 days at room temperature Filter and use.

Wright's Stain

It is marketed either as a ready made solution or in tablet form. The tablet is dissolved in acetone free methyl alcohol as per maker's instructions.

J.S.B. Stain

Solution I—Dissolve 0.5 G of Methylene blue, medicinal, in 500 ml of water and add 3 ml of 1% sulphuric acid gradually and stir the mixture thoroughly. Add 0.5 G of Potassium dichromate. This produces a purple precipitate. Add 3.5 G of Disodium hydrogen phosphate. Stir till precipitate dissolves. Boil this in a flask fitted with a reflex condenser for 1 hour. when the blue colour of the solution will become deeper. It is ready for immediate use.

Solution II—Dissolve 1 G of water-soluble eosin in 500 ml of tap water. Keeping for a week or more renders it a deep red and this is very suitable for staining. Both solutions keep well for several months.

May-Grunwald Stain

Place 0.3 G of the stain in a flask of 250 ml capacity. Add 100 ml of methanol and warm the mixture to 50°C. Allow it to cool to room temperature and shake several times during the day. Filter after 24 hours and use.

69. Preparation of alumina ($Al(OH)_3$ gel)

Pour 50 ml of ammonia solution (sp. gr. 0.88) into ml of distilled water and then add this to 600 ml of tilled water at 63°C containing 22 Gm of ammonium phate. Bring the temperature rapidly to 58°C. Stir orously and pour rapidly in one lot into this mixture olution of 76.7 Gm of ammonium alum at 58°C. Stir ten minutes without allowing the temperature to l below 58°C. Separate the precipitate by centrifug- and wash five times with 1,500 ml of distilled water, first washing with 300 ml volume of distilled water ntaining 0.44 ml of ammonia (sp gr. 0.88, diluted 1 2) and second 300 ml volume containing 0.88 ml of uted ammonia, subsequent washings with plain dis- ed water. Suspend the precipitate in the least nount of water to make a gelatinous suspension which n be pipetted. Store in glass stoppered bottle.

70. Veronal buffer (pH 7.35)

0.1 M-sodium diethyl barbiturate ...	570 ml
0.1 N-Hcl	430 ml
Sodium Chloride	5.67 Gm

Dilute the buffer with equal volume of 0.9% saline.

PART II

HISTOPATHOLOGY

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FIXATION AND FIXATIVES

roduction

71. Fixation is an essential requisite for success- cutting and staining of a tissue. Proper attention details of fixation must be devoted as no subse- ent treatment of the tissues can compensate for proper fixation.

e Aim and Effects of Fixation

72. The *objects* of fixation are

(a) to prevent putrefaction and autolysis of tissues removed from the body either during life or after death,

(b) to solidify colloid material of the cells,

(c) to harden to a degree that it will lend itself to further manipulations,

(d) to aid in visual differentiation of cellular structures; this is done by the fixative which alters to varying degree, refractive indices of the cell and tissue components.

The *effects* of various fixatives on subsequent aining must be borne in mind. This will be dealt with nder the description of each fixative. For adequate xatic n in a reasonable time the tissue should not e too thick. The total bulk of the tissue taken deter- ines the volume of fixative needed while its thickness etermines the speed of fixation. The length and readth of the tissue piece taken determines the f microtome to be employed. The best thicknes

routine use is 3—5 mm. The bulk of fixative used sh
be at least 20 times the volume of the tissue take

Reagents employed as Fixatives

73. It is essential to know the properties, m
and demerits of the common reagents employed a
or in combination as fixatives.

(a) *Formaldehyde (HCHO)*—Formaldehyde i
gas. It is soluble in water at a rate of 40%
weight. The commercial product known
"Formalin" is saturated solution of the ga
water. In making various percentages of
malin, the commercial reagent should
taken as the unit. Thus when we say 10% s
tion of formalin in isotonic saline, we m
one part of commercial formalin mixed v
9 parts of isotonic saline.

(i) *Advantages of formalin*—Easily availa
and cheap. Penetrates and fixes the tis
well. Preserves fat, myelin, nerve fib
amyloid, haemosiderin and various or
nisms. It permits a variety of staining p
cedures to be undertaken after fixat
and causes no shrinkage of tissue.

(ii) *Disadvantages of formalin*—On storage
becomes cloudy, especially in cold weath
due to the formation of a white precipita
of para-formaldehyde. Since para-form
dehyde is formed from formaldehyde, t
strength of the formalin solution will
down with storage. Slow in action. It c
cause serious dermatitis to those who cor
in close contact with its vapour and w
are allergic to it. Its vapours are irritati
to the eyes and nose and can cause sinu
tis. It is essential to wear rubber gloves
handling specimens preserved in formali

The following solution has been found to relieve the lesions caused by formaldehyde—

Urea crystals C.P.	...	5 gm
Ammonium phosphate	...	1 gm
Distilled water	...	100 ml

Formalin solution contains formic acid either as an impurity in the commercial preparation or as a product of oxidation of formaldehyde. For certain special staining procedures, fixation of tissues in neutral formalin is essential. It is inferior to alcohol as a preservative for iron and other pigments. It often changes the colour of bile concretions from yellow to green. It causes troublesome precipitation of fine crystalline brownish black pigment from laked haemoglobin.

(iii) *How to obtain neutral formalin*—The best and ideal way to obtain neutral formalin is to distil it. This is not always practicable. Any of the following procedures may be followed:—

1 Addition of borax to diluted formalin until it shows a good red colour with phenolphthalin or slaty blue with thymol blue.

2 To allow formalin to stand on a layer of calcium, sodium, magnesium or lithium carbonate. The disadvantage of using such minerals arises when alcohol is used with formalin as a fixative for microinceneration of tissues.

3 Buffer it to pH 7 by addition of 3.5 g of anhydrous sodium dihydrogen phosphate (NaH_2PO_4) and 6.5 g of

anhydrous disodium hydrogen p
phate (Na_2HPO_4) per litre of dil
fixing solution. This method of neu
lisation notably increases the
quency with which ferric iron can
demonstrated in blood pigments
also the solution does not cause
position of formalin pigment on
tissues. Add 2% calcium acetate
diluted formalin. In addition
neutralisation, it helps in the preser
tion of phospho-lipids.

(iv) *Removal of formalin pigment from s
tions*—Any of the following two tech
ques can be employed—

1 *Schridde's method*—Treat sections
30 minutes in 75% alcohol 200 ml p
25—28% ammonia water 1 ml.

2 *Verocay's method*—Treat sections
10 minutes in 80% alcohol 100 ml pl
1% aqueous potassium hydroxide 1 m

After using either of the above metho
wash the sections thoroughly in water b
fore placing them in 80% alcohol an
staining.

(v) *Action of formalin on tissues*—The actio
of formalin on tissue proteins are comp
lex and numerous. The general consensu
of opinion is that the formalin does no
precipitate proteins but forms additiv
compounds with them. It preserves som
compound lipids and has no action o
neutral fats—a fact responsible for th
ease with which, in formalin fixed mater
ial, neutral fats can be demonstrated by
frozen sections.

(b) *Mercuric Chloride* ($HgCl_2$)—It is used as a saturated solution—about 7%. It is seldom employed as a fixing agent by itself but enters as a constituent in many valuable compound fixatives. Mercury acts as a protein precipitant.

(i) *Advantages*—Very rapid in penetration and hardening of tissues. Cytoplasmic and chromatin staining is greatly improved.

(ii) *Disadvantages*—Depth of penetration is small and hence if too thick a piece of tissue—exceeding 5 mm, is put in it the periphery is overfixed while the interior is underfixed.

It allows great shrinkage of tissue because of uneven fixation. Tends to overharden the tissue if fixation period is prolonged beyond a certain limit. Hence it is called an "intolerant" fixative. Overhardening of tissues will make cutting of thin sections difficult.

It is radio opaque. Presence of mercuric chloride in calcified tissues precludes the use of X-rays to determine the end point of decalcification.

Mercury corrodes metals. Fixatives incorporating mercuric chloride must not be stored in containers with metal caps. It produces brownish precipitate in the tissues. This requires treatment of sections or blocks of tissue by iodine and sodium thiosulphate.

(iii) *Removal of mercury pigment from sections*—Place the section in 0.5% iodine in 80% alcohol for 3 minutes. Rinse in water. Place in 3% aqueous sodium thiosulphate for 3 minutes. Wash in running water for 1-2 minutes. Then proceed with staining.

(c) *Potassium Dichromate* ($K_2 Cr_2O_7$)—This on tissue proteins like formalin forming a tive compound with them without precip ting them.

(i) *Advantages*—It is a good cytoplasmic ative especially for mitochondria. Pre ves phospho-lipids.

Causes only moderate hardening. It ha mordanting action on subsequent stain procedures.

(ii) *Disadvantages*—It is a poor nuclear fi tive as it dissolves chromatin. Weake nuclear staining. Tissues preserved fixative containing chromic salts require be washed well in running water befo transfer to alcohol. Direct transfer to cohol will cause formation of insolub lower oxides which cannot be removed.

(d) *Chromic Acid*—It is prepared by dissolvi anhydrous chromium trioxide (CrO_3) in di tilled water. It is a powerful oxidising agen It precipitates proteins and preserves carb hydrates. It enters into composition of ORTH fluid and ZENKER's fluid.

(e) *Osmium Tetroxide* ($Os O_4$)—It is incorrectl known as "Osmic Acid". Osmium tetroxide i supplied in sealed glass tubes containing 0.5 o 1 g. It is used as a 2% solution. To prepar such a solution, the label on the ampule i removed and the ampule washed severa times in a glass of distilled water. A file mark having been made, the tube is then broken and dropped into a glass-stoppered dark bottle con taining the correct amount of distilled water The solution should be kept in cool dark place

as it is easily reduced by heat and light. To check reduction, add 1 drop of saturated aqueous solution of mercuric chloride to every 100 ml of the Osmium tetroxide solution.

(i) *Advantages*—It demonstrates lipids like myelin by fixing and staining them black. The blackening is due to conversion of the colourless OsO_4 to the black hydrated form OsO_2 , $5H_2O$. It does not precipitate proteins but forms additive compounds with them. Vapour is used for fixation in cytological studies as this preserves the very fine cell details in Golgi bodies and mitochondria.

(ii) *Disadvantages*—Very expensive. Poor penetration. Vapours are very irritating and can cause conjunctivitis.

(f) *Price Acid* [C_6H_3 $(NO_2)_3$ OH]—This is explosive when dry. It should always be kept under water. As a fixative it is used as a saturated solution—approx 1%. It forms an important constituent of many fixatives. It precipitates tissue proteins as picrates some of which are water-soluble. Treatment by alcohol renders the water-soluble protein picrates, water-insoluble. Hence it is essential to treat tissues fixed in picric acid containing fixative, for a period with alcohol, before transferring them to water. The trichrome stains give brilliant contrast after fixing in picric acid containing fixatives.

(g) *Ethyl alcohol*—It is seldom employed as a fixative by itself except for fixing blood smears. Alcohol penetrates slowly and tends to harden the tissue. When used with other fixatives as in Carnoy's fluid, the penetration and fixation

is very rapid. It precipitates proteins a glycogen is preserved well. It is used in his chemical methods for enzymes because some extent alcohol leaves them in their c ginal state. It dissolves fats and lipids.

(h) *Acetone* (CH_3-CO-CH_3)—Use of cold aceto is coming into the fore as a fixative in his chemical demonstration of tissue enzym notably the phosphatases and lipases. It do not preserve glycogen well.

(j) *Acetic Acid* (CH_3 $COOH$)—It is common called glacial acetic acid because it is solid temperatures below 17°C. It is used in 1—5 strength and is used as component of a fixativ and not alone. Used alone it causes swelling collagen tissue. In compound fixatives, counteracts the shrinking effect of other co stituents. It is a good nuclear fixative and poo cytoplasmic fixative. Mitochondria and Golg apparatus are destroyed or distorted.

(k) *Trichloracetic Acid*—Like acetic acid, it i never employed alone as it swells the tissues. I is a protein precipitant and has some decalcify ing properties. It is employed in a compound fixative.

Classification and Properties of Compound Fixatives

74. It is seldom that one fixative is suitable for variety of methods of staining and demonstration of various cellular or tissue constituents. From the point of view of the extent to which a fixative allows further manipulations, the compound fixatives are divided into three groups—

75. *Microanatomical Fixatives*—Fixatives for routine use should be drawn from this group. Fixatives of this group preserve the anatomy of

the tissue by maintaining the correct relationship of the tissue layers and cellular aggregates.

(i) *Formol Saline*

Formalin	...	...	10 ml.
Sodium chloride	...	...	0.9 g
Water ...	...	...	100 ml.

It is a very tolerant fixative and is perhaps the most widely used of all the fixatives. Tissues can be left in it for long periods without causing excessive hardness and can be sectioned easily after as long a period as one year. The fixation is complete in 24 hours at room temperature. For rapid fixation, pieces of not more than 3 mm thickness can be put in formol-saline heated to 55°C and kept at that temperature for 2-3 hours.

(ii) *Formol-Calcium*

Formalin	...	...	10 ml
Calcium Chloride	...	...	1 g
Water to ...	...	...	100 ml

This fixative has all the advantages of a neutral formalin with the additional property of preserving phospholipids. It is not recommended when calcium, acid and alkaline phosphatases are to be demonstrated because of the calcium content. Calcium Chloride can be replaced by calcium acetate in 2 g quantities; the acetate acts as a buffer and keeps the pH at 7. Fixation time is the same as for formol-saline.

(iii) *Heidenhein's "Susa"*

Mercuric chloride	...	4.5
Sodium chloride ...	...	0.5
Trichlor acetic acid ...	...	2
Acetic acid	..	4
Formalin	...	20
Distilled water to ...	..	100

This is probably the best fixative for r tine biopsy work. It gives brilliant stain and good cytological details. It is w balanced, and gives rapid and even pe tration with minimum shrinkage. It is ho ever, an intolerant fixative, as tissues l for long time in it become bleached a excessively hard. The trichlor-acetic a incorporated in it is said to help in decal fying small calcareous deposits. The p caution of removing mercury from t sections before staining should be take The method of removal of mercury fro sections is given under discussion on Me curic Chloride. Tissues fixed in "Sus should be transferred direct to 96% abs lute alcohol to avoid swelling of conne tive tissue. Tissues not exceeding 7-8 m in thickness are fixed in 12—24 hour Small pieces not thicker than 3 mm a fixed in 2-3 hours. It should be noted tha "Susa" ruins RBCs.

(iv) *Zenker's Fluid*

Mercuric Chloride ...	...	5 g
Potassium dichromate	...	2.5 g
Sodium Sulphate ...	...	1 g
Distilled water to ...	...	100 m

Add 5 ml of glacial acetic acid immediatel before use.

The fluid does not keep after the acetic acid has been added. The fluid without acetic acid is called "Zenker stock fluid". This is a good general fixative. Zenker fixed tissues preserve nuclear structure, bacteria and fibrils of all kinds. Zenker fixed tissues must be washed in running water overnight to remove all the excess of bichromate. Then transfer them to 70% alcohol. Fixation is usually complete in 12 hours. Small pieces, not thicker than 3 mm, are fixed in 2-3 hours. Zenker's fixation gives good results with procedures like Mallory's connective tissue stain for demonstration of tendons, Purkinje cells, muscle, fibrin and haemofuscin. Zenker's fluid inhibits silver staining.

(v) *Zenker's Formol* (*Helly's Fluid*)—The only difference from Zenker's Fluid is the addition of 5 ml of Formol—10 ml of formol in Maximow's modification, instead of acetic acid. It is an excellent micro-anatomical fixative. Unlike Zenker's acetic fiuid it preserves cytoplasmic granules well. It is an excellent fixative for bone marrow, spleen and other blood containing organs like kidney. This fluid acts slower than Zenker acetic. Time for fixation is 3—18 hours and prolonged fixation for more than 24 hours is harmful. This fluid is particularly useful if Giemsa or Leishman stains are to be employed.

(vi) *Bouin's Fluid*

Picric acid saturated aqueous solution	75 ml
Formalin (40% formaldehyde)	25 ml
Glacial acetic acid ...	5 ml

This is the most useful of all the acid containing fixatives. Tissues are fixed with no shrinkage. It gives br staining with Mason's trichrome m of staining. Giemsa's stain gives colouration of protozoal parasites afte ation in this fluid. Owing to formati water soluble picrates with pro ns, fixed tissues should directly be transf to 70% alcohol. Fixation is usually c lete in 24 hours, but small pieces no ceeding 2-3 mm in thickness are fixe 2-3 hours. It penetrates poorly and RBCs. It is not a good fixative for aut material. It preserves glycogen and h is useful for fixing liver, muscle and pernephroma. Sections fixed in Bo fluid do not adhere to the slides well.

(vii) *Gendre's Fluid*

Picric acid saturated solution in 95% alcohol	... ...	80 m
Formalin (40% formaldehyde)	... .	15 m
Glacial acetic acid	...	5 m

This is a good fixative for demonstra of glycogen. The fixation time is 3 hours.

76. *Cytological Fixatives*—When preservation intracellular structures or inclusion bec paramount. fixatives of this group are emp ed. These fixatives act at the expense of pe trability, ease of cutting and of cellular st tures other than what is intended to study.

(i) *Carnoy's Fluid*

Absolute alcohol	... ..	60 ml
Chloroform	.. ...	30 ml
Glacial acetic acid	...	10 ml

This is a very rapidly acting fixative and penetrates very quickly. It gives excellent nuclear fixation. It preserves Nissl substance and glycogen. The disadvantage is that it causes considerable shrinkage and even loss of cytoplasmic elements. Shrinkage of tissues can be reduced by allowing the fixation to go on at 0°C for 18 hours. The usual time for fixation is 1-2 hours; small pieces—2 to 3 mm in thickness are fixed in 15 minutes.

(ii) *Newcomer's Fluid*

Isopropanol	...	...	60 ml
Propionic acid	...	...	30 ml
Petroleum ether	...	...	10 ml
Acetone ...	...	...	10 ml
Dioxane ...	...	...	10 ml

This fixative is rapid in penetration, particularly useful for fixing chromosomes and preserves chromatin better than Carnoy's fluid. It improves Feulgen reaction. Fixation is complete in 12—18 hours; pieces not exceeding 3 mm in thickness are fixed in 2-3 hours.

(iii) *Champy's Fluid*

3% Potassium dichromate ...		7 ml
1% Chromic acid ...	...	7 ml
2% Osmium tetroxide	...	4 ml

This fluid should be prepared from stock solutions. Since the penetrative power is low and uneven, pieces of tissue should be thin and not more than 2 mm in thickness. It is an excellent fixative for cytological

details like mitochondria. It preserve
yolk and lipids. Before transferring
tissue to alcohol, it must be thoro
washed overnight. Fixation time for ti
not exceeding 2 mm in thickness
hours.

(iv) *Regaud's fluid*

3% potassium dichromate	...	80 m
Formalin (40% formalde-hyde)	... .	20 m

This fluid does not keep. It has to be
pared fresh every time. The penetrati
rapid and even. There is a tendency
this fluid to overharden the tissues.
fluid is particularly good for demonstra
mitochondria. For demonstrating m
chondria the tissue should be fixed in
fluid for 4 days, changing the fluid ev
day; this is followed by mordanting
which the tissue is put in 3% potass
dichromate for 7 days changing the f
every 2nd day. Regaud's fluid can be
ployed for demonstrating chromoffin
action. Addition of 5% acetic acid impro
reaction. For routine work fixation
complete in 24 hours: tissues not exc
ing 3-4 mm in thickness are fixed in 4
hours.

(v) *Muller's fluid*

Potassium dichromate		... 2.5 g.
Sodium Sulphate	...	... 1 g.
Distilled water to	...	... 100 m

Prolonged fixation and mordanting is
quired with this reagent. This fluid
originally employed for nervous tiss
but it is now seldom used except for

study of bones in which case the bone is fixed in this fluid for 3 months followed by decalcification in 3% formic acid—formalin. This fluid can also be used for post-chroming.

(vi) *Schaudinn's fluid*

Mercuric chloride saturated aqueous solution	...	2 parts
Absolute alcohol	...	1 Part

This can be recommended only for fixing wet smears in the study of amoebae. Addition of glacial acetic acid enough to give 1 per cent solution improves results. This fluid is not recommended for tissues as it is an intolerant fixative and penetration is low. Wet smears require 10—20 minutes fixation. If the smear is too thick, it is likely to float off the slide during fixation.

77. *Histochemical fixatives*—When it is desired to study certain specific chemical constituents or enzymes of a cell, it is essential that fixatives used should cause as little change as possible in the elements to be studied. For this the ideal method will be freeze-drying. But it is too troublesome and time consuming for routine work and moreover it will not be practicable in all the laboratories. Certain less elaborate methods of fixation for histochemical work only will be described.

(i) *Formol saline*—Since most tissues are likely to become fixed in this common fluid it is fortunate that many histochemical methods can still be employed on such material. In employing histochemical methods on formalin fixed tissues it is well to remember to employ neutral formalin and the tissue is well washed off the formalin.

(ii) *Cold Acetone*—Immersion of tissues cold acetone (0-4°C) is recommende enzyme studies especially phosphatas

(iii) *Absolute Alcohol*—Sections cut by fr drying technique can be fixed in abs alcohol for 24 hours. Because formalin ed tissues permit most of histo-chem examinations, absolute alcohol is sel now employed.

Choice of a Fixative

78. From the foregoing description of the meri various individual fixatives, it is clear each one of them has some advantages off by disadvantages. To get the maximum be fit of the advantageous properties of the f tives, the agents are employed in comb tion. Such fixatives are called compound f tives. To choose a compound fixative fron large list of different types, one should guided by the types of investigation requi both immediately and in the future. For ample there is no use fixing a tissue in C noy's fluid with the immediate object studying the chromosomes if later on a ne arises to demonstrate lipids in the same tiss In such cases. it is a wise precaution to p serve the major part of a tissue in a tolera fixative like formol saline and use small pe tions of the tissues for fixing in a specialis way for studying certain constituents. It mu be remembered that museum specimens which colour must be restored can be prepa ed only from formalin fixed material.

Practical Guide to Choice of Fixative

79. It is difficult to give general rules because th choice depends on the nature of the elemen to be demonstrated and the stains to be em ployed.

Autopsy tissues ..	Formol Saline Sublimate (F.S.S.) solution or Helly.
Small cellular biopsies	Susa or F.S.S.
Large surgical specimens	Fix whole in formol saline, select blocks and refix them for 8-18 hours in F.S.S. or Helly or Bouin. This fixation is a great improvement but is a luxury rather than a necessity.
Tissue for frozen sections	F.S.S. is possible but difficult.
Nervous system ..	Fix in formol saline. Select blocks and if desired refix these for paraffin embedding in F.S.S. For myelin or for silver staining, tissues are fixed in formol saline only.
) Haemopoietic tissue—	
Marrow	Helly
) Glycogen	Bouin, Carnoy or alcohol.

DEHYDRATION, CLEARING AND IMPREGNAT

Since paraffin is not mixable with water an almost insoluble in alcohol, water must be comple removed from the tissues with upgraded st engths alcohol and the alcohol is removed by a "cleari agent before paraffin wax impregnation.

80. **Method for routine material**

(a) 95 per cent alcohol—overnight.
(b) Absolute alcohol—4 hours.
(c) Chloroform or xylol (see note (iii) below 4 hours.
(d) Saturated solution of paraffin wax in xylol chloroform at 37°C—overnight.
(e) Paraffin wax at 56°C with one change in hours.

81. **Method for research material—Zenker fix tissue**

(a) 50 per cent alcohol—4 hours.
(b) 80 per cent alcohol—4 hours.
(c) 95 per cent alcohol—overnight.
(d) Absolute alcohol with one change in 8 hours
(e) Chloroform—overnight.
(f) Equal parts of chloroform and molten paraffi wax at 56°C—12 hours.
(g) Paraffin wax at 56°C with one change in hours.

NOTE.—(i) Include a typewritten label with the seria number in the vessel containing the tissue. This accompanies the specimen as it is transferred from vessel to vessel.

(ii) During dehydration and clearing, gently blot off the excess fluid from the tissue between coarse filter papers each time the tissue is transferred. During impregnation and casting, handle the tissues with forceps warmed just to above the melting point of the paraffin.

(iii) Clearing—Cedar wood oil is the best, although a most expensive clearing agent. Specimens thus cleared require several changes in the paraffin bath until no odour of cedar wood oil is perceptible. Chloroform is superior to xylol but the process takes longer. If the clearing agent turns milky when the tissue is placed in it, dehydration is incomplete and must be repeated.

(iv) Paraffin bath: In temperate climates use wax of 50°—55°C M.P; in the tropics a harder wax of 55°—56°C M.P. must be used, the temperature of the embedding oven being adjusted accordingly. If the wax is dirty on supply, filter in the oven. Stock wax is kept in a glass or enamel vessel in the oven. Small glass jars are suitable for impregnation of tissues.

(v) Material containing much muscle, fibrous tissue or blood should be cut as thin as possible and should not have more than 4 hours in the paraffin bath. Small pieces of skin e.g. leprosy clips should not be left in absolute alcohol for longer than 4 hours.

82. **Embedding**

Type of mould—Metal L moulds are slid together on a piece of glass so as to form a box appropriate to the size of the specimen. Boxes made from stiff paper

Do not press hard and continue till all
have disappeared. When stropping, drav
knife over the strop edge last, in an ob
direction so that the entire length of the
is stropped. Do not press hard. At the er
each stroke turn the knife over. Knives in
condition will require only slight strop
See that no dust collects on the hone and
fore stropping c ean the knife with a soft
moistened with xylol. A good quality mac
oil or liquid paraffin is used as lubricant
the hone: a commercial "dressing" for
strop is advantageous.

85. **Procedure**

Mark slides with the serial number of the b
and arrange them on a rack in the order in which
proposed to cut the b ocks. Remove the paper box f
the block or disengage the L moulds. Place the b
so that the surface to be cut lies face upwards. T
the block by downward paring with a scalpel until
sides of the object are surrounded by a cube or r
angle of wax for a distance of 2-3 mm. The upper a
lower surfaces of the block must be parallel in re
tion to the microtome knife edge. If necessary, pare
surface of the block until the object b gins to app
in the shavings. Heat the handle of a metal scalpel a
melt the wax of the under surface of the block. Pr
the latter against the chuck of the microtome a
place the chuck and attached block face downwards
the ice tray of a refrigerator or on a block of ice a
leave for 5 minutes. This is usually unnecessary
temperate climates but is essential in the tropics. F
the chuck on microtome so that the upper and low
surfaces of the block are parallel to the knife edge. A
just the thickness indicator to 12 microns and cut se
tions until the whole surface of the object is in conta
with the knife edge. Move the knife so as to bring
fresh portion of its edge in relation to the block ar

the indicator to the desired section thickness, ally 7 microns is adequate for routine purposes. h rocking microtomes a fairly rapid, rather jerky vement of the handle is usually advantageous. h the rotary pattern do not turn the handle too wly. Hold the free end of the section ribbon with a r of forceps and after about six sections have been free the attached end with a needle. At this stage sections will be crumpled. Float the ribbon into a dish of water heated to the correct temperature t warm enough to the hands and flatten the sec- ns by applying gentle traction to the ends of the bon with a pair of needles. It may be necessary to d more hot water to deal with wrinkles on individ- sections. Separate the ribbon into portions of a nvenient size, at the line of junction of individual tions and float the sections on the slides. Stand the des to drain and transfer them to an oven at 50°— C for two hours and then to an incubator at 37°C ernight.

86. Errors in section cutting (after H.M. Carleton)

Fault	*Cause*	*Remedy*
bon is curved	(*i*) Upper and lower surfaces of block not parallel.	(*i*) Retrim the block
	(*ii*) If one border of section is crumpled the knife edge is dulled at that point	(*ii*) Use another segment of the knife
ribbon formed	Wax is too hard	Rub the finger several times along the surface of the block and cut sections quickly
tions are fragmented	(*i*) Wax too soft	(*i*) Return block to refrigerator for few minutes
	(*ii*) Knife blunt	(*ii*) Restrop knife
tions longitudilly scratched	(*i*) Knife edge serrated	(*i*) Rehone
	(*ii*) Debris from sections on knife edge	(*ii*) Clean knife by passing it between thumb and finger

Fault	*Cause*	*Remedy*
Sections cut sometimes thick and sometimes thin	(*i*) Feed mechanism badly adjusted	
	(*ii*) Knife improperly clamped	Correct the fault
	(*iii*) Chuck loose	
Width of section less than that of block with result that sections will not flatten	Bevel of knife lost	Rehone

If the object is so brittle that all the sections a fragmented, wipe the surface of the block with 1 collodion or with "surgical" collodion. Blow on t block for few seconds and cut a single section. If d attention has been paid to the above points in sectio cutting and satisfactory sections are not produced, t processes of dehydration, clearing and impregnatio must be carefully checked. Hard, but non-calcifie tissues should have a preliminary soaking in 4% aq eous solution of phenol for 1—3 days. The thinne possible slides should be used so that the time in t paraffin bath can be cut down.

87. **Frozen Sections**

Where a freezing microtome is not available, pe fectly satisfactory frozen sections can be prepared b the following method.

(a) *Fixation*—as in routine methods for paraffi sections, or select portion of tissue and trim t 15×15×5 mm.

Bring about 15 ml of 10 per cent formol saline t the boil in a wide test tube, insert the tissue and leav the test tube in the embedding oven for 10 minutes.

(b) *Equipment*—Microtome, any pattern. Chuck preferably of adjustable type. Wooden block holde can be made by cutting a 3/8 in. cross section from a

ce of wood. This is trimmed to fit the chuck. If the face of the latter is plain, the block holder is fixto it with molten wax, which is allowed to cool roughly before use. If the chuck is recessed, the ck holder is fitted into it by similar means. Ethyl oride in spray bottle. 4 inch petri dish. Large flat n as used for flattening paraffin sections. Glass dles prepared from glass rod. Camel hair brush. % alcohol in distilled water.

(c) *Procedure*—The 4 inch petri dish is almost ed with 40% alcohol and the large dish filled with d tap water. Roughly adjust chuck and block der parallel to knife; then remove. Adjust knife in der and clamp. A little water is put on the surface the block holder so as to aid in the secure attachnt of the tissue. The tissue is carefully but firmly ssed into the block holder with the forefinger and yl chloride is sprayed on and around it for a few conds. Remove the finger and re-spray the tissue, king sure that the part covered by the finger is well zen. The material should now be firmly fixed to the ck and ready to cut. Insert the chuck and cut secns as rapidly as possible at a thickness of 5—15 crons. Re-spray at short intervals when cutting wn to a particular part of the tissue. The cut secns are gently wiped off the knife edge with the mel hair brush and are placed in the 40% alcohol tri dish for a few minutes. They are then transferred the surface of the tap water using the glass needle, hen it will be found that the alteration in surface nsion causes them to be flattened. Float the sections to albuminised slides as for paraffin sections and and until almost dry. Pass the slide with section upards rapidly through a flame in order to coagulate e albumin. The sections are now ready for staining routine methods and for mounting in Canada lsam. Sections for fat staining are left in the 40% cohol for 5 minutes and then treated according to e method of choice.

DECALCIFICATION

88. Bone and calcified tissues are sawed into 2—5 mm thick before fixation. A fret saw or jewe saw should be used so as to damage the tissue as as possible. Thorough preliminary fixation and ening are essential; any nonacid fixative may be 10 per cent formol saline being suitable.

Procedure

Transfer the tissues to 5% nitric acid in 60% cohol. This fluid must be used in large amounts. tissue should be suspended in a gauze bag so that dissolved salts may sink to the bottom of the ve Decalcification should not be prolonged beyond days, the fluid being changed several times. Compl ness of decalcification can be judged by gently pr ing the tissue with a needle; in most instances tissues will have become flexible in 24—48 hours. T wash off every trace of acid with several changes 60% alcohol during 24 hours and proceed with de dration.

Bone marrow and tissues containing only delic bone trabeculae become perfectly decalcified dur Zenker fixation owing to the action of the acetic ac This method has the added advantage that cellular tail is preserved far better than by the acid alcoh technique necessary for dense bone. In cutting se tions of decalcified tissue, discard the first half doz or so, which will show laceration due to the sawing.

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STAINING

General

(a) Frequently used stains and reagents should be kept in stoppered jars—the coplin pattern is the most suitable. For stains and other reagents in infrequent use, drop bottles should be suitable. Haematoxylin solutions should be filtered daily when in use, other staining solutions weekly. Grooved porcelain slide dishes are helpful for washing purposes. Volatile substances must not be kept in open jars or dishes.

(b) A supply of neutral distilled water must always be available. This is conveniently kept in a bottle, aspirator connected to a jet and pinchcock.

(c) If a good quality "rectified spirit" is available it is wasteful to prepare lower grade alcohol from absolute alcohol.

Table of dilution of 96% alcohol

Grade required	Volumes of rect. spt.	Volumes of aq. dest
90%	93·5	6·5
80%	83·5	16·5
70%	72·9	27·1
60%	62·5	37·5
50%	52·1	47·9
40%	41·6	58·4
30%	31·2	68·8

(d) When using stains and other reagents drop bottles, place two lengths of glass r ross the sink, about $1\frac{3}{4}$" apart and faster the bench with plasticine or place the plastic stands.

(e) The degree of staining and differentiation always be controlled under the micros For most purposes a $\frac{2}{3}$ in. objective and eyepiece are used.

(f) Coverslips must be kept in absolute alc and dried with a soft cloth before use. I slips are boiled for a few hours in sulph acid-bichromate mixture, washed thorou in running tap water, rinsed in distilled w and transferred to absolute alcohol. If an immersion lens is to be used a No. 1 1/250 in. coverslip is absolutely necess Dirty slides are cleaned in the same way.

90. Theory of staining—after H.M. Carleton

(a) The stains usually employed are extracts plant and insect tissue, (e.g. haematoxy from the bark of a tree and carmine from insect) or aniline dyes. For certain purposes salts of the heavy metals are selectively pre pitated over particular tissue components, silver nitrate. Stains are coloured in soluti and are taken up by the tissue: impregnat agents are colourless in solution and are duced in particulate form over the tissue e ments.

(b) *Basic, acid and neutral stains*—Most stains a salts. A basic stain is a salt, the base of whic contains the colouring substance, combine with an acid, usually inorganic, e.g. basic fuc sin comprises the base rosaniline combine with the acid radicle Cl. An acid stain is on in which the acid component of the dye mole cule is the coloured one, the basic componen

being colourless e.g., acid fuchsi nis the sodium salt of a sulphonic acid derivative of rosaniline. A neutral stain is formed by the mixture of aqueous solutions of certain acid and basic stains.

(c) *Progressive and regressive stains*—A progressive stain is one which slowly stains tissue elements in a definite sequence, e.g., Ehrlich's haematoxylin stains first the nucleus and then the cytoplasm. A regressive stain, most commonly employed, involves the deliberate overstaining of the material and then the differentiation of the various tissue elements, e.g., haematoxylin followed by acid alcohol.

(d) *Mordants*—Most aniline dyes stain the tissues in a simple aqueous or alcoholic solution. Many stains, especially haematoxylin require a mordant, e.g., a substance which makes possible or enhances the staining process. Potash alum and iron plus ammonium alum are thus used in certain haematoxylin staining methods.

(e) *Metachromasia*—Most stains affect the tissues in varying degrees of the same fundamental colour. Certain tissues, when treated by a particular stain, assume a colour different from the fundamental colour of the stain, e.g. thionin is a blue dye which stains most tissue elements in varying shades of this colour but the ground substance of cartilage, mucin and basophilic granules stain purple. Methyl violet stains nuclei and cytoplasm in shades of blue while amyloid is stained red.

(f) *Specific stains*—Some stains have a special affinity for certain tissue elements, e.g., orcein stains elastic tissue black, Scharalach R stains intracellular neutral fat and other lipids red, acid fuchsin stains collagen fibrils red.

(g) Staining is a complex process which probab involves both chemical and physical proces and is influenced by fixation. In a few insta ces, notably in the "staining" of fat, the d molecules are more soluble in the fat than the solvent. In some cases, such as the affin for basic dyes shown by chromatin and "bas philic" granules, there may be an actual chem cal combination owing to inherent acidity these structures. Often, however, absorption a companied by electrical phenomena seem likely to play an important part in staini processes.

91. **Routine procedure for paraffin sections**

(a) *Removal of paraffin wax*—Transfer the sli into two changes of xylol, 3 minutes eac wipe off the excess of xylol in between.

(b) *Removal of xylol*—Wipe off the excess of xyl and transfer to absolute alcohol for 1 minu In the case of post mortem material containi much blood, place the slide in 2% caustic so in absolute alcohol for 2 minutes, wipe off t excess and transfer to absolute alcohol for minute.

(c) Transfer to rectified spirit for 1 minute.

(d) Transfer to tap water, unless distilled water specified for subsequent staining, or direct the stain, if alcoholic.

(e) Proceed with staining as described below unle the tissue has been Zenker fixed, when the mc curic oxide deposit must first be removed described under Zenker fixation.

(f) *Dehydration of the stained sections*—Flood th slide with rectified spirit for 30 second followed by absolute alcohol for 1 minute.

(g) *Clearing*—Treat with xylol for 1 minute. This removes the alcohol and raises the refractive index of the tissue.

(h) *Mounting*—Place a drop of Canada balsam in xylol on the section and gently lower on the coverslip so as to avoid bubbles. If these form, they can often be got rid of by gently tapping the coverslip. Do not use too thick a solution of balsam. Lay the slide flat and allow the balsam to set; this can be accelerated by placing sections in the embedding oven. For delicate work take the sections down to water through absolute, 95% and 70% alcohol and dehydrate in the reversed sequence. This is however unnecessary for routine work.

92. **Ehrlich's haematoxylin and eosin**

Haematoxylin	...	2 gms
Absolute alcohol	...	100 ml
Glycerol ...	...	100 ml
Distilled water	...	100 ml
Glacial acetic acid	...	10 ml
Potash alum	...	3 grs

Dissolve the haematoxylin in the alcohol and add the other ingredients. Ripen by exposure t and sunlight in a paper capped vessel, for 6 w shaking the bottle daily to accelerate the oxidatio

Staining

1. Take sections down to water in the u manner.
2. Transfer to Ehrlich's haematoxylin for 5 minutes according to the "ripeness" of particular batch of stain.
3. Rinse off the excess of stain and transfer gentle stream of tap water until the secti become blue.
4. Examine under the microscope. The nuc should be dark blue—if insufficiently stain replace sections in the haematoxylin. Differe tiate in 10 per cent solution of HCl in absolu alcohol until the maximal nuclear detail apparent; this usually takes only a fe seconds. Rinse in tap water until the sectio are blue once more.

5. Transfer to 1 per cent aqueous solution of eosin for 45 seconds.

6. Rinse rapidly in water, dehydrate and clear. Eosin is rapidly extracted by low grade and more slowly by high grade alcohols. With practice the length of staining and duration of dehydration can be modified to suit individual requirements.

Results

A well stained preparation should show: nuclei e; acidophil nucleoli red and basophil nucleoli blue; dophil cytoplasm, muscle and collagen fibrils pink; l blood cells bright pink; eosinophil granules vivid dish pink.

Weigert's iron haematoxylin and Van Gieson— for collagen fibrils or connective tissue

1 Take sections down to water.

2. Stain in Weigert's haematoxylin 2.5 to 5 minutes.

3. Blue section in tap water—do not leave sections too long in tap water.

4. Counterstain with Van Gieson's stain for 1½ to 2 minutes.

5. Pour off the stain and blot.

6. Dehydrate quickly in absolute alcohol and blot.

7. Clear in xylol and mount.

Van Gieson stain

Picric acid 1.22 per cent ...	...	5 ml
Acid Fuchsin 1 per cent solution ...		0.75 ml

This stain does not keep and must be made up esh on each occasion.

Results

Nuclei should be greyish-black; collagen bright red; epithelium, muscle and red blood yellow.

94. Gomori's method—for reticulin fibrils

Reagents

1. Potassium permanganate 1 per cent sol
2. Potassium or sodium meta bisulphite cent solution.
3. Ferric ammonium alum 2 per cent solut
4. Formaldehyde 15 per cent solution in water.
5. Stock gold chloride 1 per cent solution. Fo prepare 0.2 per cent solution. This 0.2 per gold chloride will not keep.
6. Sodium thiosulphate 2 per cent solution.
7. Silver nitrate 10 per cent solution.
8. Potassium hydroxide solution 10 per cent
9. Ammoniacal silver nitrate solution prep as under:—

   Take 12 drops of silver nitrate 10 per cent s tion in a test tube and add 3 drops of 10 cent potassium hydroxide solution. Add str ammonia drop by drop while shaking until precipitate is completely dissolved. Add a silver nitrate cautiously till the solution ta on a very fine sheen. Make the solution v distilled water to twice the volume. This be used for 2 days.

Staining method

1. Bring sections to tap water as usual.
2. Oxidise with 1 per cent $KMnO_4$ for minutes. Rinse in tap water.

3. Decolourise in 3 per cent sodium metabisulphite for 1 minute.
4. Sensitise in 2 per cent ferric ammonium alum for 1 minute, wash in tap water for few minutes followed by two changes of distilled water.
5. Impregnate with ammoniacal silver nitrate solution for five minutes.
6. Rinse gently in distilled water for 5 to 10 seconds.
7. Reduce in 15 per cent formaldehyde for 3 minutes. Wash in tap water for a few minutes.
8. Tone in 0.2 per cent gold chloride solution for 10 minutes. Rinse in distilled water.
9. Reduce toning in 3 per cent sodium metabisulphite for 1 minute.
10. Fix in 2 per cent sodium thiosulphate solution for 1 minute.
11. Wash in tap water, dehydrate, clear and mount in the usual manner.

Results

The finest connective tissue fibrils are sharply delineated in black and a heavy blackish deposit outlines the cell nuclei. These fibrils wil not take specific stains for collagen and silver impregnation must be employed when assessing the amount of connective tissue stroma and its relation to individual cells or cell groups in sarcoma, reticulo-sarcoma, endothelioma and anaplastic carcinoma. The differential diagnosis of these tumours is usually impossible without recourse to silver impergnation.

NOTE—When using silver impregnation methods, the sections tend to float off the slides unless handled very gently. Flood the sections with the various solutions using a pipette and do not squirt the solution directly on the section. Wash the sections in a slide dish using a very gentle stream of running water.

95. **Weigert's Resorcin Fuchsin stain—for elast**

Staining solution

Basic fuchsin (Gurr or BDH)	...			2
Resorcinol	...	...	...	4
Distilled water		...	...	200

In a large porcelain dish place the distilled and heat. While hot add the other ingredients a well with a glass rod and bring the solution While briskly boiling, add 25 ml of 29 or 30 p ferric chloride solution little by little stirring b additions. Continue boiling for 2 to 5 minutes

A thick copious black precipitate separates. to cool and filter. Discard the filtrate. Place the glass rod and filter paper with precipitate in the bator at 37°C overnight for complete drying.

Add little by little 200 ml of 95 per cent a and cautiously heat: when precipitate on the paper has dissolved, remove filter paper, cool filter. Make up to 200 ml with 95 per cent alcohol 4 ml conc. hydrochloric acid and preserve in the gerator.

The solution is used in a Koplin jar.

The stain keeps only for 2 to 3 months, after w it loses its selectivity.

Staining

1. Bring sections down to water.
2. Rinse in distilled water and place in the st ing solution in a Koplin jar for 1 to 2 hour
3. Decolourise till the background is clear w 95 per cent alcohol.
4. Place in running tap water for 1 hour.
5. Counterstain the nucleus lightly with May watery haematoxylin for $2\frac{1}{2}$ minutes.
6. Blue section in water.

7. Quickly rinse in 95 per cent alcohol, dehydrate, clear and mount.

Results

Elastic laminae stain deep bluish black, nucleus ht blue.

Gram Weigert's method for Gram positive organisms

1. Fixation—formalin or zenker.
2. Cut sections at 4 μ
3. Take sections down to water.
4. Stain nucleus lightly with Mayer's watery haematoxylin for 5 minutes.
5. Blue in water.
6. Place in 2.5 per cent aqueous eosin at 56°C for 15 to 30 minutes.
7. Rinse in water.
8. Filter on to the section Sterling's aniline crystal violet and allow to act for 30 minutes.
9. Wash well in water.
10. Apply Lugol's iodine for 2 to 3 minutes.
11. Wash in water and blot well.
12. Decolourise with aniline-xylol in equal parts till section is pink. Blot.
13. Apply 3 changes of xylol and mount in balsam.

Results

Gram positive organisms deep blue, nucleus light ue and other elements pink or red. By this method ram negative organisms are not stained.

The aniline crystal violet gives better staining than mple aqueous methyl or crystal violet.

Sterling's aniline crystal violet

Crystal violet	... ...	5 gm
Absolute alcohol	...	10 ml
Aniline	... ...	2 ml
Water distilled	... ...	88 ml

Filter before use.

Lugol's iodine

Iodine	... ...	1 gm
Pot. Iodide	... ...	2 gm
Water distilled	...	100 ml

97. **Weigert's differential stain for fibrin**

Fixation

Fix in absolute alcohol, Carnoy's fluid or formalin. Zenker and Helly's fluids may also b but after fixation in fluids containing chrome sa sections must be treated with potassium perman 0.25 per cent solution for 10 minutes and oxalic per cent solution for 20 minutes before stainin bed, preferably in paraffin.

Staining solution

Solution A

Absolute alcohol	... ...	33 ml
Aniline	... ...	9 ml

Methyl violet (crystal violet)—in excess

Solution B

Methyl violet (crystal violet) saturated tion in distilled water (about 1.68 per ce

Just before use, mix 3 ml of solution A with of solution B. The stock solutions will keep indefi the combined stain for only 1 to 2 weeks.

Method of staining

1. Stain nuclei by the lithium carmine meth the usual way and wash thoroughly in w

2. Stain sections for 5 to 10 minutes in the above staining solution.
3. Drain and blot with filter paper.
4. Pour over the sections Gram's iodine solution and allow it to act for 5 to 10 minutes.
5. Drain off solution and blot with filter paper.
6. Differentiate in a mixture of equal parts of aniline and xylol from a dropping bottle. Blot and pour on fresh aniline-xylol several times until the section is well differentiated and no more purple colour is removed.
7. Blot and pour on xylol several times to remove the aniline.
8. Mount in balsam.

Results

Fibrin and Gram positive bacteria—blue to blue-lack; nuclei—red.

3. **Giemsa stock solutions**

Azure II Eosin	3 gms
Azure II	0.8 gm
Methyl alcohol acetone free	375 ml
Glycerine neutral	125 ml

Grind little by little and make up volume and lter.

Working Giemsa solutions

Giemsa stock solution	3 ml
Methyl alcohol acetone free	3 ml
Neutral distilled water (add 2 drops of 1 per cent sodium bicarbonate to 100 ml dist water)	100 ml

Method

1. Bring sections down to distilled water.
2. Place in dilute Giemsa solution, chan solution twice during the day and leav third change overnight.
3. Wash in distilled water.
4. Differentiate in 95 per cent alcohol to w few drops of Colophonium in absolute a have been added.
5. Dehydrate with two applications of ab alcohol.
6. Clear and mount as for Leishman's stai

Results

Nucleus of cells and neutrophil granules—vio red; Basophilic granules—blue—violet; cytoplas lymphocytes—blue; acidophilic granules and RE pale red.

99. **Leishman's stain—for paraffin sections**

1. Fixation—formalin, Zenker or Susa.
2. Cut paraffin sections and take sections to tilled water.
3. Apply Leishman's stain diluted 1 in 2 to 3 in distilled water for 15 to 20 minutes.
4. Wash in distilled water.
5. Differentiate in dilute acetic acid (4 to 5 dr of 1/300 acetic acid added to 100 ml of tilled water).
6. Blot and quickly pass through one appli tion of 95 per cent alcohol and blot.
7. Apply absolute alcohol twice and blot.
8. Clear well in xylol and mount in neut Canada balsam or better in Gurr's neut mounting medium. Mount in cedar wood for best results.

Results

Nuclei—bluish red; acidophil granules—pink to ; basophil granules—blue; red blood cells—salmon-.

Ziehl Neelsen's method—for staining of Acid Fast Bacilli

NOTE—1. This stain tends to be less acid fast than in sputum.

2. No heating of the stain on the slide as the sections char.

1. Fixation—formalin.
2. Cut sections at 4 μ
3. Remove paraffin by treating with two changes of the following mixture: —

   Xylol=2 parts.

   Cotton seed or peanut or olive oil=1 part.

   Blot well to opacity.
4. Filter on to the section Ziehl Neelsen's carbol fuchsin and allow to stain for 30 minutes. The stain should cover the whole slide, otherwise precipitate will form in the sections.
5. Wash thoroughly in water.
6. Decolourise with 1 per cent Hcl in 70 per cent alcohol till the section is almost colourless.
7. Wash well in water.
8. Apply 1/10 of 1 per cent methylene blue for 5 minutes or less.
9. Wash in water. The sections should be only slightly blue.
10. Slant the slide and when completely dry, mount in plenty of Canada balsam.

Results

AFB—red and background—light blue.

The above method works equally well for both ercle and lepra bacilli.

101. **Fite-Faraco technique for staining Lepra Ba**

1. Deparaffinize sections with two changes mixture of 1 part of cotton seed, peanu olive oil and 2 parts of xylol.
2. Drain, wipe back and sides and blot se with filter paper.
3. Treat with (aqueous) Lugol's iodine solu and with 5 per cent sodium thiosulphate tion to remove mercury precipitate fron Zenker solution.
4. Wash in water.
5. Stain for 15—20 minutes in Ziehl Neel carbol fuchsin at room temperature.
6. Decolourise for 1-2 minutes to a faint colour with 1 millilitre of concentrated hy chloric acid in 99 millilitres of 70 per alcohol.
7. Wash in tap water.
8. Counterstain with Mayer's alum haematox for 2-3 minutes or alternatively, with G aqueous methylene blue (1 per cent) ½ minute.
9. Wash in tap water till blue.
10. Decolourise with above acid alcohol if n sary (few seconds).
11. Blot with filter paper and keep in incubat 37°C over night.
12. Mount in clarite or similar synthetic med

Results

Myco-lepra—red; Nuclei—blue.

102. **Macchiavollo's technique modified for Ricket and inclusion bodies**

Method

1. Bring paraffin section to water.
2. Stain in 0.25 per cent aqueous basic fuc for 30 minutes.

3. Differentiate rapidly (about 3 seconds) in 0.5 per cent citric acid.
4. Wash in tap water.
5. Counterstain for 15—30 seconds in 1 per cent methylene blue.
6. Wash in water.
7. Dehydrate, clear and mount in Canada balsam for DPX.

Results

Rickettsiae, inclusion bodies—red; Tissue elements ue.

103. **Seller's stain for Negri bodies in smears**

Solutions

1. Saturated solution of methylene blue (2 gms/ 100 ml) in methyl alcohol acetone free.
2. Saturated solution of basic fuchsin (4 gms/ 100 ml) in methyl alcohol acetone free.

Method

1. Mix 15 ml of the saturated alcoholic methylene blue with 25 ml of methyl alcohol and then add 2 to 4 ml of the basic fuchsin solution.
2. While still wet, the smear is stained for 5 seconds washed in water and air dried.

Results

The Negri bodies are exceptionally brilliant red th blue granules.

. **Benhold's staining for amyloid**

1. Fixation—formalin.
2. Cut frozen sections 5—10 μ straight away after washing in water for 24 hours or after gelatin embedding.

3. Float sections in distilled water minutes.
4. Place sections in 1.0 per cent aqueous red (filter before use) for ½ hour. Used red solution can be added to stock sol
5. Place sections for 30 to 45 sec. in sat aqueous lithium carbonate to mordar stain.
6. Wave the sections in 80 per cent alco remove the excess of stain (till no clouds of stain come out).
7. Float in distilled water for 15 minutes.
8. Stain the nucleus for 1 to 4 hours in 1 ir 1 in 6 Mayers watery haematoxylin di in distilled water.
9. Blue the sections in a dish of tap water.
10. Mount in glycerine jelly or preferably advantage of permanency, dehydrate, and mount in balsam.

Results

Nucleus—blue; Amyloid—rosy red.

105. **Fat staining**

1. Fixation—formalin.
2. Cut frozen sections at 5 to 10μ after wash the piece of tissue for 24 hours in runn water or after gelatin embedding.
3. Float sections in water. If they do not unfu pass through 40 per cent alcohol and refl in water. Another method is to dip the fing in 40 per cent alcohol before collecting t section from the knife and floating in wat
4. Float in 40 per cent alcohol, the sectio should be flat and there should be no a bubbles below or above the sections. Whe ever the alcohol does not come into conta

with the section, even if fat be present there, very poor staining results. Leave for ½ hour.

5. Carefully transfer to scarlet R saturated solution in 70 per cent alcohol—70 per cent alcohol is brought to the boil, remove from the flame and add little by little scarlet R taking care not to allow too much frothing. Usually about 0.2 gm are required for 100 ml. The solution can be used only after 24 hours, as its immediate use results in plenty of precipitate on the section. The solution is filtered for use and stored at room temperature. (Krajain advises 1 gm of benzoic acid to be added to 100 ml of the solution as it not only intensifies the staining property but also preserves it). Stain for ½ hour. The sections should be flat without any air bubble in a closed staining box.
6. Rapidly decolourise in 40 per cent alcohol and float in water.
7. Counterstain nucleus with 1 in 4 Mayer's watery haematoxylin for about an hour.
8. Blue section in dish of tap water.
9. Take an albumenised slide and mount in glycerine jelly.
10. Allow to set in the refrigerator, scrap off excess of jelly and seal off the edges with thick copal varnish.

Results

Fat—depending on the nature of the fat, dirty brown to ruby red; Nucleus—blue.

NOTE:—Scarlet red solution once used should be discarded. No dehydration, clearing and mounting in balsam is possible as fat is soluble in alcohol.

106. **Masson's trichrome stain for connective ti**

1. Fixation—formalin, Bovin or Regaud's

2. Embed in paraffin and cut sections 4 thick.

Staining solutions

(i) Regaud's haematoxylin

(ii) Differentiating solution for the nucleus ric alcohol).

Alcohol 95 per cent saturated with picric —2 parts (about 7 per cent).

Alcohol 95 per cent—1 part.

(iii) *Solution A* (for tissues other than connect

Acid fuchsin	...	0.3 gm.
Ponceau de xylidine	...	0.7 gm
Distilled water	...	100 ml
Glacial actic acid	...	1 ml

(iv) *Solution B* (Mordant and differentiator)

Phosphomolybdic acid	...	1 gm
Distilled water	...	100 ml

If the phosphomolybdic acid is of a variety t does not readily go into solution, place 1 gm of phosphomolybdic acid into 50 ml of N/10 sodi hydroxide, shake well to dissolve. Add little by lit 50 ml of N/10 hydrochloric acid. Store in the dark f 24 hours and filter. Store in dark bottle.

(v) *Solution C* (for connective tissue)

Glacial acetic acid	...	2 ml
Distilled water	...	100 ml

Aniline blue (water soluble)—To saturation

Boil water and add 2.3 gms of aniline blue. whil warm add acid and allow to cool. Filter.

All solutions are stored in the refrigerator.
For the picric alcohol, dilute solution is made
n saturated alcoholic picric acid before use.

Method

1. Take sections down to water.
2. Mordant with 5 per cent ferric ammonium alum previously heated to 45°C to 50°C in a test tube—5 minutes. Wash thoroughly in water.
3. Filter on to the section Regaud's haematoxylin and stain for 5 minutes on the top shelf of the paraffin incubator.
4. Pour off stain and rinse in 95 per cent alcohol.
5. Differentiate nucleus in picric alcohol. Place in running water till all the picric acid is washed off. Examine under the microscope, only the nucleus should be stained and the background should be colourless. If further differentiation is required, blot the section, rinse in 95 per cent alcohol and differentiate in picric alcohol.
6. Wash thoroughly in running tap water.
7. Stain in filtered solution A for 5 minutes.
8. Rinse in distilled water.
9. Differentiate in solution B for 5 minutes.
10. Pour off, *do not wash*, filter on to the section 10 drops of solution C. Allow to act for 5 minutes.
11. Rinse in distilled water.
12. Differentiate in Solution B again for 5 minutes.
13. *Do not wash*, place 1 per cent acetic acid for 5 minutes.
14. Do not wash, dehydrate, clear and mount.

Results

Nucleus—blue to black; Collagen—intense other structures, cytoplasm, neuroglia fibrils—ve lion red; argentaffil granules—black or red.

The Masson's trichrome method is superior to Gieson in its stability and bringing out finer detai

107. Iron pigments

1. Fixation—formalin.
2. Embed in paraffin and cut sections.
3. Take sections down to distilled water.
4. Pour on the slide the following mixture allow to act for ½ hour:—

   10 per cent potassium ferrocyanide ... 2 pa
   20 per cent Hcl ... ... 1 pa
5. Wash in distilled water.
6. Counterstain with 1 per cent neutral red 1 or 2 minutes.
7. Wash in distilled water.
8. Differentiate in 95 per cent alcohol, deh drate, clear and mount.

Results

Iron pigments—blue against a pale red bac ground.

NOTE—

1. The reaction depends upon the formation a ferro-ferri-cyanide complex—prussian blu which is blue in colour, but the iron tissues is usually found in a combined sta with the protein. The liberation of free iro is due to the hydrochloric acid. However, th treatment with acid cannot liberate the iro found in haemoglobin.

2. If the acid is poured first followed by the ferrocyanide, the blue colour is diffuse; hence the section is treated with the mixture.
3. Extraneous contamination with iron is to be carefully avoided. This contamination may be due to:—
   (a) Fixing in iron containers.
   (b) Using formalin stored in drums.
   (c) Little particles of the metal detached from the knife.
   (d) Using rusted iron implements.

108. Levaditi's method for spirochaetes

1. Fix small pieces in formalin (preferably neutral formalin).
2. Place in running water for 6 hours.
3. Place in 95 per cent alcohol for 24 hours for hardening.
4. Place in distilled water and wait till the tissue sinks to the bottom.
5. Place in fresh 1.5 to 3.0 per cent aqueous silver nitrate at 30°C for 3 to 6 days (for biopsy material 3 per cent silver nitrate is preferable and for autopsy material 2 per cent silver nitrate solution is preferable).
6. Wash in distilled water for 2 to 5 minutes.
7. Reduce for 24 to 48 hours in the dark at room temperature in the following solution:

Pyrogallic acid	3 gms
Commercial formalin 40 per cent ...	5 ml
Distilled water	100 ml

8. Wash in 3 changes of distilled water for a total of 15 minutes.
9. Place in 70 per cent, 80 per cent, 90 per cent, 95 per cent alcohols for 12 to 24 hours each.

10. Complete dehydration, clear and e
paraffin.
11. Cut thin section.
12. Remove paraffin and mount in balsa

Result

Back ground—light yellow or yellowish
Spirochaetes—jet yellow.

109. **Vonkossa's method—for calcium deposit**

The method affords only an indirect test
presence of calcium. Most of the calcium is pr
the form of phosphates and carbonates. The
solutions used do not react with the calcium i
therefore do not give a positive test for the p
of calcium. It is only the presence of the silve
phate seen in the section that gives an indica
the presence of the calcium because the phosphat
carbonates are mostly those of calcium.

Method

1. Fixation—formalin (neutral formalin is
ly recommended). Any trace of acid will
to be avoided.
2. Embed in paraffin and cut sections.
3. Bring sections down to distilled water.
4. Place the slide with the sections up in a
dish and pour enough 5 per cent aqueous
ver nitrate to cover the section. Leave ou
exposed to direct sunlight for 1 hour.
5. Wash thoroughly in distilled water.
6. Place in 5 per cent thiosulphate solution
3 to 5 minutes.
7. Wash thoroughly in distilled water.
8. Counterstain in 1 per cent aqueous safra
for about 2 minutes.

9. Wash in distilled water dehydrate, clear and mount.

Results

Nucleus—red; calcium—black.

10. **Papanicoloau stain for cytological examination of smears for cancer cells**

The vaginal or other material is obtained with a pecial pipette and placed on clean slides. The smears re made and immediately before drying are placed or fixation in a mixture of equal parts of 95 per cent lcohol and ether. The slides are left in this solution n the refrigerator and can be stained after about 6 ours. The slides may be allowed to remain for about 0 days in the refrigerator though it is not advisable o do so.

If the slides have to be mailed, the fixed smear s taken out of the solution and before it is dry a arge drop of glycerine is placed on the smear. Another lide is placed over it and slightly pressed for the glycerine to spread all over. In this condition the slides may be allowed to remain for about two weeks.

Staining solutions

1. Harris haematoxylin.
2. O.G—6

   Orange G(0.5 per cent in 95 per cent alcohol) —100 ml.

   Phosphotungstic acid (Merck)—0.015 gm.

   Alcoholic solution of orange G is first prepared by heating, as orange G is less soluble. Addition of phosphotungstic acid intensifies the colour. The solution is not filtered.

3. *EA* 36

   Light green SF yellowish (0.5 per cent in 95 per cent alcohol)—45 ml.

Bismark brown (0.5 per cent in 95 per cent alcohol)—10 ml.

Eosin Y (0.5 per cent in 95 per cent alcohol —45 ml.

Phosphotungstic acid (Merck)—0.2 gm.

Lithium Carbonate—1 drop.

(Saturated aqueous).

All the stains should be of National Aniline Chemical Company. The alcoholic solutions are first prepared separately using heat and then filtered. They are then mixed and other ingredients are added. All solutions are preserved in the refrigerator when not in use. Solutions can repeatedly be used.

If glycerine had been used, remove glycerine with 95 per cent alcohol.

Staining method

1. Alcohol 80 per cent—5 dips.
2. Alcohol 70 per cent—5 dips.
3. Alcohol 50 per cent—5 dips.
4. Alcohol 50 per cent—5 dips.
5. Distilled water—5 dips.
6. Harris haematoxylin—10 minutes' wash in tap water—10 dips.
7. Hydrochloric acid 0.5 per cent—5 dips. No more.
8. Running tap water—5 minutes.
9. Distilled water—5 dips.
10. Alcohol 50 per cent—5 dips.
11. Alcohol 70 per cent—5 dips.
12. Alcohol 80 per cent—5 dips.
13. Alcohol 95 per cent—5 dips.
14. Alcohol 95 per cent—5 dips.
15. 0.G.6—45 seconds to 1½ minutes.
16. Alcohol 95 per cent—5 dips.

7. Alcohol 95 per cent—5 dips.
8. EA 36—1½ to 2 minutes.
9. 95 per cent alcohol—5 dips.
0. 95 per cent alcohol—5 dips.
1. 95 per cent alcohol—5 dips, blot.
2. Absolute alcohol—2 minutes, blot.
3. Absolute alcohol and xylol equal parts—2 minutes, blot.
4. Xylol 1,2,3—2 minutes each.
5. Mount in balsam.

Results

Acidophilic cells—red to orange; basophilic cells een or blue-green; cells penerated by blood—ge or orange-green.

Note

Smears of urine sediment, gastric and bronchial rates, pleural and peritoneal fluids, the fluid after ration, is immediately mixed with an equal volume 5 per cent alcohol and centrifuged; smears are le out of the deposit which are fixed as per the inal smears. Or, the supernatant is removed from sediment and is covered with absolute alcohol and ed in the refrigerator for sometime. Make smears usual. If a proper smear cannot be made, slides ted with egg albumen may be used.

Sputum smears

Sputum is collected directly in 70 per cent alcohol. mall quantity of the lump is placed on albumenis-slide, spread and fixed. Do not crush too much with ther slide. The use of 70 per cent alcohol is for the rpose of prevention of bacterial contamination. The e of alcohol also shows a better cell structure. The advantage is that we do not get many cells, but the vantages outweigh the disadvantage.

111. **Warthin-Starry's method for paraffin sectio for spirochaetes**

Use Walpole's buffer ie 18.5 ml of a solutio 11.8 ml acetic acid in 1000 ml distilled water 1.5 ml of a solution of 16.4 gm of sodium aceta 1000 ml of distilled water, the mixture giving a of 3.6.

1. Bring sections down to distilled water pH (20 ml of the buffer added to 480 ml of tilled water).
2. Impregnate for 45 minutes at 56°C in 1 cent Silver nitrate prepared in the buffer tilled water (prepared above).
3. Place slides on glass rods and pour on developer previously heated to 56°C.

Developer

Bring to boil 1000 ml of the buffered disti water, crowd in 5 gms of gelatin; remove from flame and stir well to mix. To 15 ml of this gel solution, add just before use 3 ml of 2 per cent sil nitrate and 11 ml of 3 per cent hydroquinone, b these solutions having been prepared in the buffe distilled water. While developing, avoid direct s light. Develop for 3 to 5 minutes till the sections come golden-brown or the developer starts to t black.

4. Pour off the developer rinse with tap wa at 60°C and then with plain distilled water
5. Dehydrate, clear and mount.

Results

Spirochaetes—jet black; background—yellow brown.

112. **Mallory's phosphotungstic acid—Haematoxyl method (Anderson's modification)—for neurog fibres**

1. Fix in formaldehyde saline.
2. Embed in paraffin or celloidin.

3. Cut sections at 8—10 μ
4. Wash thoroughly in distilled water.
5. Transfer to saturated mercuric chloride aqueous solution for 20 minutes.

N.B.—With Zenker-fixed material omit this step.

6. Wash quickly in distilled water, and place in Anderson's neuroglia mordant for ten minutes.

   Anderson's mordant

   Distilled water—100 ml.

   Sodium sulphite—5 ml.

   Oxalic acid—2.5 gm.

   Pot. iodide—5 gm.

   Iodine crystals—2.5 gm.

   Dissolve in the above order and then add 5 ml of glacial acetic acid. Keep in a well stoppered bottle.

7. Without washing, transfer to Lugol's iodine for 15 minutes.
8. Wash in two changes of 95 per cent alcohol.
9. Wash well in distilled water and transfer to 0.25 per cent potassium permanganate for 15 minutes.
10. Wash well in distilled water and transfer to 5 per cent oxalic acid until bleached white.
11. Wash in two changes of distilled water and stain in well ripened phosphotungstic acid haematoxylin for 18 to 24 hours at room temperature.

    Haematoxylin ammonium—0.1 gm.

    Distilled water—100 ml.

    Phosphotungstic acid crystals—2 gms.

Dissolve the haematoxylin in a little water b warming; when cool and water to 100 ml the add the acid. Ripen by adding 10 ml of 0.2 per cent potassium permanganate solution.

12. Without washing transfer to 95 per cen alcohol for one to two minutes. Dehydrat clear and mount in balsam.

Results

Neuroglia and fibrin—Blue.

113. **Cajal's Gold Chloride—Sublimate method fo Astrocytes**

1. Cut frozen sections at 15 to 30 μ from materia fixed in formalin for at least 14 days.
2. Wash rapidly in distilled water a few times.
3. Treat with 10 per cent ammonia water for 2 hours at room temperature or for 4 hours a 37°C.
4. Wash in distilled water twice.
5. Place in a 10 per cent solution of hydrobro- mic acid and let the sections remain for 2 to 4 hours.
6. Pass twice quickly through distilled water to which a few drops of ammonia are added.
7. Transfer to the following solutions for 3½ to 4 hours:

Pure gold chloride 1 per cent soln. ..	6 ml
Mercuric chloride 5 per cent soln. ..	6 ml
Double distilled water	35 ml

The solution should be freshly made and the staining to be done in a cool dark place. Successful staining depends upon the tempe- rature. Early in the morning, with doors and windows opened, is perhaps the best time (18 to 20°C).

8. Wash in ample distilled water.
9. Treat with the following mixture—

5 per cent Sodium thiosulphate	...	40 ml.
95 per cent alcohol	... ...	10 ml.

Place sections in this solution for 5 to 10 minutes and add 2 to 3 drops of saturated sodium bisulphite over it.

10. Wash in two changes of 40 per cent alcohol.
11. Dehydrate through 70 per cent, 90 per cent and absolute alcohols.
12. Clear in carbol-xylol, then in xylol, and mount in balsam.

Results

Astrocytes with their processes—black;

Background—almost unstained or light brownish purple;

Nerve cells—pale red;

Nerve fibres—unstained.

4. Bielschowsky's Silver Impregnation for Neuroglia Fibres

1. Fixation—formalin upto 14 days. Freshly fixed and shortly fixed tissues are washed in running water for 24 hours. Longer fixed tissues are washed for 48 hours. Cut frozen sections 5 to 10 μ thick.
2. Preparation of impregnation solution—Use pure chemicals and pure distilled water. The glassware should be chemically clean. In a cylinder with a glass stopper place 10 ml of 10 per cent silver nitrate. Add 5 drops of a fresh 40 per cent aqueous NaOH or KOH and mix thoroughly. Add distilled water, invert a few times, allow to stand for the precipitate to settle. Pour off carefully the supernatant fluid.

Repeat washing with distilled water 3 to
times till the supernatant fluid is crystal clea
Pour off carefully the supernatant fluid. It i
important to retain as much of the precipitat
as possible. Add liquor ammonia fortis dro
by drop till the precipitate is just dissolve
The amount of ammonia should not exceed 1
to 20 drops. Make up the volume to 20 ml wit
distilled water. This solution is made and use
on the same day.

Method

1. Rinse sections in two changes of distilled wate
and leave in a third change of distilled wate
for 1 to 2 hours. From hereon handle the sec-
tions only with glass needles.

2. Transfer to pure pyridine for 24 hours. Wash
thoroughly in distilled water. This step can be
safely omitted without ill effects.

3. Impregnate in 2 per cent aqueous silver nitrate
solution for 24 hours in the dark.

4. Rapidly carry sections through 2 changes of
distilled water.

5. Place sections in impregnating solution till the
sections become dark seal brown. This usually
takes from 10 to 20 minutes.

6. Wash rapidly in 3 changes of distilled water.

7. Place in 20 per cent formalin made with tap
water for 10 minutes for reduction.

8. Place in tap water for 15 minutes.

9. Rinse in distilled water and tone sections in
Gold Chloride (add 3 to 5 drops of 1 per cent
Gold Chloride Solution to 10 ml distilled
water and to this solution add 2 drops of
glacial acetic acid).

While toning, sections are examined under the microscope. When the neurofibrils stand out, the toning is complete. This may happen in a period of anything between 20 minutes and 6 hours.

10. Rinse in distilled water and fix in 5 per cent thiosulphate for 2 minutes.
11. Place in tap water for 2 hours.
12. Take on albumenised slides, dehydrate with 95 per cent and absolute alcohols.
13. Clear first in carbol-xylol (1 part of carbolic acid and 9 parts of xylol), then in xylol and mount in balsam.

Note

1. It is very important that while carrying sections with glass needles, solutions should not get contaminated with the preceding solution. Therefore use of a number of glass needles is advised.
2. To minimise the time of toning, use 2 per cent gold chloride with 3 drops of acetic acid for every 10 ml. The toning will be complete in about ½ hour. Acidified gold chloride gives a violetish background with black neurofibrils standing out.

   If a greyish background is desired use 10 ml of 2 per cent gold chloride to which a few drops of dilute lithium carbonate have been added. Instead of gold chloride a solution of chloroplatinic acid slightly acidified may be used.
3. All solutions, for staining purposes are placed in clean petri dishes.

Results

Neurofibrils—greyish black to deep black; background—pale violet or grey.

This method is an excellent one to stain axis cy
ders and dentrites, and in well stained thin secti
the intra cellular neurofibril net will be seen as a d
cate meshwork of dark brown or black lines. The a
cylinders and their processes and the muscle p
termini in striated muscle are stained intensely bl
The neuroglia fibres are very faintly stained and
be seen only with difficulty except where there
been marked proliferation, as in the neuroglia scars
tabes, or multiple sclerosis. Hence they can be seen
fine, usually closely packed wavy lines coloured a d
cate lavender or purple. Longitudinal sections of
cord in multiple sclerosis stained by this method sh
to excellent advantage, the axis cylinders intact w
their medullary sheaths replaced in neuroglia fibr

115. **Modified Holmes—for Axons**

Special solutions required:—

(1) Buffer solutions

(*a*) Boric acid A.R.	12·4	gms
Distilled water .. .	1000	ml
(*b*) Borax ($Na_2B_4O_7$ $10H_2O$) A.R. ..	19	gms
Distilled water	1000	ml

(2) Reducer

Hydroquinone	1	gm
Sodium sulphite crystals	10	gms
	(or 5 gms an	
	drous sal	
Distilled water	100	ml

The reducer can be used repeatedly, but m
not keep for more than a few days.

(3) 20 per cent aqueous silver nitrate solution

Procedure

The blocks of tissue are embedded without m
danting; longitudinal sections are usually the mo
informative and are cut at about 8 μ though for so
purposes, as in the study of end organs, thicker sectio

necessary. They are mounted on albumenised and dried overnight at 37°C. On the following the slides are placed for an hour in the paraffin to complete the flattening and drying.

1. The slides are taken down to distilled water which can b eused once only. Take 55 ml of silver nitrate in the dark at room temperature. where they remain for 1 hour but upto 2 hours is permissible; the solution may be used repeatedly so long as it does not contain a black deposit of silver.

2. Meanwhile prepare the impregnating solution, which can be used once only. Take 55 ml of the boric acid buffer solution. Mix these in a 500 ml measuring cylinder and dilute to 494 ml with distilled water. With a pipette add 1 ml of a 1 per cent aqueous solution of silver nitrate (AR) and with another pipette 5 ml of a 10 per cent aqueous solution of pyridine.

3. Take the slides from the 20 per cent silver nitrate and wash for 10 minutes in 3 changes of distilled water.

4. Place the slides in the impregnating solution (not less than 20 ml of solution per slide) in covered glass vessels of a kind which can be kept thoroughly clean.

5. Impregnate at 37°C until any convenient time on the following day.

6. Then take out the slides, shake off the superfluous solution, and place them in the reducer described above for not less than 2 minutes.

7. Wash in running tap water for 3 minutes; the slides should not lie back to back during the process or traces of reducer will be carried over. Rinse in distilled water at this stage until it is convenient to proceed.

8. Tone in 0.2 per cent aqueous gold chlori (yellow) for 3 minutes (the gold chloride m be used repeatedly provided it does not co tain the brown clouds indicative of reductio
9. Rinse briefly in distilled water.
10. Place in 2 per cent aqueous oxalic acid. T slides remain in this solution for 3 to minutes and are examined at intervals und the staining microscope. When the axons a thoroughly blue-black the process is stoppe further treatment may result in a diminuti of the contrast.
11. Rinse in distilled water and transfer to a 5 p cent aqueous solution of Sodium Thiosulpha for 5 minutes.
12. Wash in tap water for 10 minutes, rinse distilled water, dehydrate, clear and mount.

Results

Axon cylinders—blue black against unstain background.

Notes

1. No special precautions are necessary with t glassware, but all pipettes and vessels shou be rinsed with distilled water after use a reserved for silver impregnation by th method. Any film of deposited silver on t vessels should be removed. If the silver sol tion becomes cloudy at any stage of prepa tion, it is contaminated and should be rejecte
2. The impregnating solution described abo consists of a 0.002 per cent solution of silv nitrate containing 0.1 per cent of pyridine, a buffered at pH of 8.4. Variation in the pyridi and silver concentration is permissible a may be advantageous. Thus a heavier a sometimes more complete impregnation is o tained by increasing the silver concentrati to 0.01 per cent and the pyridine to 0.2 p cent.

In general an increase in the pyridine concentration leads to a more specific but perhaps less complete axon stain and an increase in the silver concentration gives a heavier deposit.

3. The method has given good results with material fixed in Bouin, Carnoy and similar mixtures. When these fixatives are used the axon impregnation is sharper than with formol material, but it is a disadvantage that portions of the specimen cannot be reserved for a myelin stain.

Hotchkiss-McManus Stain—For Fungus (Periodic Acid—Schiff (PAS) technique)

1. Fix specimen, dehydrate, embed and section.
2. Deparaffinise, pass through absolute alcohol.
3. Wash in distilled water.
4. Immerse in 1 per cent periodic acid for 5 minutes.
5. Wash in running tap water for 15 minutes.
6. Stain in Schiff's reagent for 5 to 15 minutes.
7. Transfer directly to two changes of either of the following for 5 minutes during each change:—

(a)	10 per cent Pot. metabisulphite ...	5 ml
	N Hcl	5 ml
	Distilled water	100 ml
(b)	Thionyl chloride	5 ml
	Distilled water	100 ml

8. Wash in running tap water for 5 minutes to 15 minutes.
9. Counterstain lightly with light green.

Light green	0.5 gm
Glac. acetic acid	0.5 ml
Dist. water	50 ml

10. Dehydrate, clear and mount.

Results

Fungi—brilliant red; background green.

Schiff's reagent

Dissolve 1 gm of basic fuchsin in 400 ml of boiling water. Cool to 50°C and filter.
To the filtrate add 1 ml of thionyl chloride. Stand in the dark for 12 hours. Clear by shaking for 1 minute with 2 grams activated charcoal. Filter. Store in the dark at 0 to 4°C. Use in the dark at room temperature.

NOTE—Since the fungi are supposed to have a covering of carbohydrate, the conversion of this into an aldehyde by treatment with periodic acid is possible. After conversion the PAS reaction can be applied to demonstrate the aldehyde.

117. Masson's Silver Method—for Melanin

The argentaffin cells of the intestine also give this reaction. This method differs from other silver methods in that no reducer is used here. Reticulum stains are too rapid to demonstrate melanin.

Masson's method

1. Fixation—any, avoid chromates.
2. Paraffin sections—distilled water.
3. Leave overnight in Fantana's silver solution in the dark at room temperature.
4. To 20 ml of 10 per cent $AgNO_3$ add strong ammonia till a few granules of the precipitate remain. Add 20 ml distilled water.
5. Rinse in distilled water.
6. Fix in 5 per cent hypo; wash well.
7. Counterstain with Carbol Safranin or Neutral red for skin sections.
8. Dehydrate, clear, mount in balsam.

Results

Melanin—black; background—counterstain.

3. **Silver-nitrate technique for melanin—Quick method**

1. Bring the sections down to distilled water.
2. Place in 2 per cent aqueous silver nitrate for two hours.
3. Wash in distilled water.
4. Place in saturated aqueous solution of sodium thiosulphate (hypo) for thirty minutes.
5. Wash and counter-stain faintly with pyronin methyl green for one minute.
6. Wash, dehydrate, clear and mount.

Results

Melanophores and melanoblasts show the presence f brownish black granules.

AUTOPSY TECHNIQUE

119. External Examination of the Body

The body should be examined in day light an attention directed to the following points:—

(a) Apparent age, sex, height, general nutrition.

(b) Presence of discolouration or pigmentation oedema or subcutaneous emphysema.

(c) Presence of any external wound or other sign of violence (together with powder marks o burns).

(d) Presence of any deformity.

(e) Presence of hernia.

(f) Condition of eyes, nose, mouth, ears, urinary meatus, anus, umbilicus, vagina, and hymen.

(g) Presence of any or all of the signs of death.

120. Internal Examination of the Body

Stand on the right side of the body. Extend the chin of the body with the left hand, and, entering the knife just above the prominence of thyroid cartilage, make a median incision downwards to the pubes. In the neck, the skin and subcutaneous tissues only should be divided; over the sternum, the incision should go down to bone, and, in the abdomen, the peritoneal cavity should be cautiously opened, the escape of any gas or fluid, or adhesion of viscera or omentum to the anterior abdominal wall, being noted during the process. The retraction of the edges is facilitated by dividing the recti subcutaneously on each side.

Now proceed to reflect the soft parts from the chest wall by everting the edge of the upper part of the abdominal incision with the left hand and by cutting

ıg the costal margin through the peritoneum. The st wall is then stripped from below upwards, every- g being reflected outwards from the costal carti- es and ribs. Traction with the left hand is of great stance. Reflexion of the soft tissue should be extend- o outer end of the clavicle on either side and to the er border of the sterno-mastoid in the neck. Make ıort examination of the peritoneal cavity before pro- ding further. Lift out the coils of small intestine and mine the pelvis, the flanks, and appendix region. te the height of the diaphragm. It should extend to fourth rib on the right and the fifth on the left. lpate the liver and spleen. The condition of the bony racic cage should now be examined. Also incisions ould be made through the mammary gland from its ep aspect, radiating from the nipple, but not pene- ting the skin. The axillary glands should be exa- ned in the same way, subcutaneously.

The thorax should now be opened by cutting rough the costal cartilages just internal to their ıctions with the ribs, and disarticulating at the rnoclavicular joints. In some cases the use of the w may be necessary. The sternum and attached stal cartilages can then be removed in one piece, ter the attachment of the diaphragm to the lower stal cartilages has been divided.

Now examine the anterior mediastinum and the eurae, noting especially presence of fluid, gas, adhe- ons, or amount of retraction of the lung. Pass the and over the whole surface of each lung in turn and ring it forwards towards the mid-line, breaking down ny adhesions gently with the fingers. Open the peri- ardium, and examine in the same way, passing the and into the sac, and lifting the heart forwards by its pex.

The right auricle should then be opened by a nall vertical incision between the superior and nferior venae cavae, and the right ventricle by an ncision from the auricular ventricular groove to the

apex along the margin of the heart. From the apex, t
cut should be extended upwards to the pulmon
artery keeping close to the inter-ventricular septu
The pulmonary artery should be examined for embo
(antemortem thrombus) and post-mortem clot. An e
bolus consists of a thrombus formed before death
some vessel, dislodged therefrom, and impacted in
pulmonary artery or its branches, or occasionally
the right auricle or ventricle. When sudden death h
resulted from pulmonary embolus, the lung, or lun
affected shows no change. An antemortem thromb
has the following characteristics. It is firm, friable, a
dry, is laminated or mottled, lies in the vessel and m
be coiled. It can sometimes be unravelled to show t
form of a cast of some other vessel. A postmortem c
in the same situation has the following characteristic
it is of a jelly-like consistency, elastic, moist, and hon
geneous; it is not attached to the wall, but forms
accurate cast of the vessel and its branches. It can
easily removed. If formed quickly, such a clot will
uniform red in colour. If clotting has taken place mo
slowly, the upper parts will be without red corpuscl
yellowish and translucent ("chicken-fat clot"). T
lower part of such a clot contains all the red blood co
puscles and is red. Should there have been an excess
leucocytes in the blood before death, the upper pa
may be milky-white. If an antemortem thrombus
found in the pulmonary artery, search must be mad
through the veins, during the rest of the examinatio
for the vessel of origin. The veins of the pelvis, leg, ar
abdomen are the commonest sites. It may be difficult
impossible to find the point of detachment, but son
roughening of the lining of the vessel may be visib
or a portion of thrombus of similar character may b
still in place adherent to the vessel wall. Both ante
mortem thrombus and post-mortem clot may be pre
sent in the same vessel.

Now proceed to remove the thoracic viscera
continuity with the tongue, fauces, larynx, trachea, an

phagus. With the blade of the knife in the upper of the original median incision, dissect the skin of neck back to the sterno-mastoid, and upwards to ramus of the jaw; reflect the submaxillary gland kwards with the skin. The floor of the mouth is next ufixed near the sympysis menti, and the tongue, n the floor of the mouth, freed, by cutting on each close to the jawbone. Hook the tongue out of the sion with the left forefinger and, with the point of fe, cut along the junction of hard and soft palates downwards on each side outside the pillars of the ces and tonsils, so that these remain in continuity h the soft palate. Cut the carotid vessels at the base the skull. Now draw the root of the tongue forward d separate the posterior pharyngeal wall, with the otid vessels, from the anterior surface of the bodies the vertebrae. This can be continued down to the racic inlet. The inner ends of the clavicles can now drawn forwards. The neuro-vascular bundles pass- into the arms should be divided as far outwards as sible. The mass of thoracic viscera can then be erated by a few touches of the knife, aided by the t hand pushing them forward from behind. The ta, oesophagus, inferior venacava and the base of pericardium are next divided and the viscera moved. If an analysis of the stomach contents is cessary, the oesophagus must be ligatured before ng divided. The thoracic organs should be laid aside further examination, and attention turned to the rd palate, naso-pharynx, jaws, submaxillary and vical glands.

The abdominal viscera should then be removed. The thods of procedure may have to be modified to meet dividual cases. Cut the small intestine across at the odeno-jejunal junction and remove the small intes- e by cutting through the mesentery with a knife ee to the gut. On reaching the caecum, proceed, with ssors to cut through the peritoneum, etc., close to gut, and free the whole length of the large intes- e. Cut through the rectum as low in the pelvis as

possible. Next turn the mesentery upwards and, with a few snips of the scissors, expose the duodenum. Open the duodenum as far as the papilla of Vater. Squeeze the gall-bladder to make sure there is no obstruction to the flow of bile. Insert a finger through the foramen of Winslow, and cut through the structures in the lesser omentum. Remove the spleen. Continue to dissect up behind the duodenum and pancreas. Remove the stomach, duodenum, and pancreas together. Remove the liver from the body.

Next dissect out the suprarenals. Remove the kidneys with the ureters attached. Should there be any disease of the urinary apparatus, the kidneys, ureters and bladder should be removed in continuity.

The various organs should then be examined in detail.

The thoracic viscera are investigated first. Place them with the posterior aspect upwards, the tongue towards you. Divide the soft palate with scissors, slit up the pharynx and oesophagus, and examine. Examine and incise the tonsils and tongue. Slit up the larynx, trachea and main bronchi. Next examine the lungs. They should be palpated and inspected and finally incised from the convex border to the root, with further incisions at right angles to this. A small piece of lung should be thrown into water, when it will float unless collapsed or consolidated.

The heart and great vessels should next be examined. Enlarge the incision made in the right auricle and inspect the tricuspid valve. Join the cut made in the right auricle with that already made in the right ventricle, and examine the right chambers of the heart. Open the left auricle with a cut between the pulmonary veins. Open the auricular appendix. Remove all clots and inspect the mitral orifice from above. It should admit two fingers. Cut from the left auricle to the apex of the left ventricle. Palpate the aortic valve with your fingers, and then cut upwards from the apex

eft ventricle (keeping close to the interventricular um) towards the aortic opening. Pass one blade of scissors through the opening and extend it, cutting ss the pulmonary artery just above the pulmo- valve. Continue the cut up the aorta and down thoracic aorta. Open up the large vessels. Remove clots and inspect interior of the left ventricle, Incise the myocardium and note its colour, con- ncy and thickness. Lay open the coronary arteries inspect them. Examine the walls of the aorta and t vessels.

Examine and incise the thyroid and thymus glands the lymph glands at the bifurcation of the hea.

The abdominal viscera may now be examined. The nentary canal must be washed out and laid open ough its whole extent. It is usual to start with the ll intestine and proceed in the order given for the oval of the viscera from the body. The intestine is ned by drawing it over the blunt end of the intesti- scissors and cutting along the line of the mesen- c attachment. The ileo-caecal valve and the appen- should be carefully examined. The wall of the gut uld be cleansed with running water, palpated, and pected along its whole extent. The stomach is open- along the greater curvature and spread out. The een is examined for adhesions and enlargements weighed. It is incised in its long axis towards, but right through, the hilum. The liver and gall blad- are separated from the rest of the viscera, if this not already been done. Weigh the liver, inspect and pate it. Turn the liver upside down and slit up and h out the gall bladder. Examine any enlarged nds in the portal fissure. Cut into the liver, now ced with its convex surface upwards, with a series ncisions parallel with the falciform ligament and ut an inch apart. These should not completely de the liver. The pancreas should next be incised its main ducts slit open.

Examination of the pelvic viscera should next proceeded with. The position, mobility, and the con tion of the vessels and ureters will have been no before their removal from the body. The kidneys m be weighed and incised from convex border to hilu with one sweep of the knife. The interior of the kidn should be examined and the capsule stripped w forceps. A catheter should be passed into the blad and any urine present drawn off for analysis. T condition of urethra, penis, and scrotum is investig ed in situ. The testes can be drawn up out of scrotum and examined and removed, if necessary. T presence of hernial sacs and congenital anoma should be noted. The bladder and rectum are of examined in situ first, and only removed if there evidence of disease or abnormality. The whole pel contents may be removed intact within a sheath peritoneum and pelvic connective tissue. The b add prostate, vesiculae seminales, urethra, and rectu should be examined in order. In the female, it is adv able always to remove the uterus, tubes and ovari and incise them carefully after removal.

In special circumstances, examine for signs recent delivery before doing this. The abdominal, pel and perineal signs should be looked for at this poi The other signs will have been noted earlier in t examination and others will be found when the pel organs have been removed and examined. The sig may be summarised as follows. The abdominal wa especially in women who have borne children, may thin and lax, and lineae albicantes are usually prese There may be pigmentation of the skin about the u bilicus and in the median abdominal line. Tears of t perineum and bruising of the vaginal wall may present. There may be a bloodstained discharge in t vagina. The os uteri will be patent and the lips swo len and perhaps lacerated and torn. The uterus is e larged and the wall thickened and vascular. T cavity is enlarged and may contain blood-clot. T

ntal site usually shows a darker ragged area, in nearly all cases has a distinctly necrotic arance, with adherent blood clot. One of the es will contain a well developed corpus luteum.

f signs of recent delivery be present and crimi- bortion be suspected, special care should be taken xamining the abdomen and pelvic organs. Perito- should be looked for and, if present, its exact and extent should be noted. Search for penetrat- vounds from the vagina into the peritoneal cavity etroperitoneal tissue. Note the extent, course etc. ined placenta and sepsis of the uterus may be ent. In such cases, if no local injury or sepsis is d, the urinary tract may show signs of conges- and inflammation such as might be caused by can- ides or turpentine, or the gastro-intestinal tracts s of irritant poisoning, cantharides, lead. The ns and contents should be kept for chemical nination.

The Head—The hair is parted in a line joining the toid processes, passing over the vertex and the scalp sed down to bone in this line. The two scalp flaps then reflected, the front one down to the supraci- y ridges, the back one to below the external occipi- protuberance. The parotid gland can be examined, ecessary, by continuing the coronal incision down- ds in front of the ear. The skull cap is then remov- by sawing.

First cut with a knife through the temporal mus- just above the scalp flaps and expose the bone. y through the outer table of the skull on either side, tinuing the saw cut across the frontal bone. From posterior ends of the lateral saw cuts, just behind above the ears, other cuts are then made through occipital bones. These cuts slant towards the vertex meet one another in the middle line just in front he occipital protuberance. In sawing through the er table, the same cuts are followed. Care must be en not to damage the meninges. If fracture of the

skull is suspected, the saw should be used until t
inner table is cut through. In other cases, when t
inner table is nearly sawn through, insert the chisel
the cut and by tapping with the mallet the remainc
of the inner table can be cracked.

The dura can be incised along the line of the s
cut, starting at the frontal region, detaching the fa
cerebri from the crusta galli and turning the dura ba
over the posterior scalp flap. If the dura is so adhere
as to prevent the skull cap being lifted off, it (the dur
should be carefully incised and removed with the bor
The skull cap should be examined, then the dura, su
dural, and arachnoid spaces.

In cases where it is necessary to remove the bra
and spinal cord in continuity, the spinal cord shou
next be exposed. The body should be placed in t
prone position. The operation is facilitated by placi
a block under the chest. An incision through the sk
and soft tissue is made in the midline, from the occip
tal protuberance to the sacrum. The spines of the ve
tebrae being thus exposed, the muscles are dissected
either side, to lay bare the laminae of the vertebra
The laminae are then divided with the saw. The spin
of the vertebrae and laminae, held together by t
ligaments, can then be cut free, opening the spin
canal. The spinal cord and its ensheathing dura shou
be examined in situ and then removed cutting t
spinal nerves as they pass from the canal. Starting
the cauda equina and working upwards, the fre
cord and membranes can be raised in the left hand
the operation proceeds. The margin of the foram
magnum should be removed by saw cuts through t
occipital bone and the cord and membranes can
drawn upwards after the cranium is opened.
remove the spinal cord separately, the membran
and cord may be cut in the cervical region.

The brain is removed by raising it gently from t
frontal lobes backward, dividing the nerves succe
sively as they appear and incising the tentorium cer
belli along its attachments. If the cord is not require

ched to the brain stem, cut across the cord as low as n be reached. If the brain and spinal cord are to be oved together, ease the brain backwards out of the of the skull, cut the dura round the foramen mag- and then draw up the cord with its membranes, oving the brain as this is done. The brain and spinal should be laid aside for further examination. It is n advisable to delay such examination until the erial has been fixed in formol saline for some days. nwhile examine the base of the skull and the ends he nerves. Lay open the cranial sinuses by incisions ough the dura; strip the dura from the bone.

The pituitary should be removed by cutting away posterior clinoid processes with the chisel. Care- y dissect the gland out of the fossa. The middle ears be exposed by removing the tegmen tympani with chisel and mallet. The mastoid air cells are best osed from the exterior through an extension of the lp incision. A complete examination of all the ac- ssory nasal sinuses can be made by removing the or of the anterior fossa of the skull. Removal of the ital plate permits of examination of the contents the orbit.

Frequently the methods of examination of the ain must be modified to suit the special features of e case. The routine procedure will be described. After amining the vessels, nerves etc., at the base, place the ain base downwards and gently separate the cere- al hemispheres with the point of a long knife (the cision of the brain tissues is easier, if the blade of the ife is wet), divide the corpus callosum throughout length, first on one side and then on the other. The int of the knife should slant slightly outwards so at the lateral ventricle is opened and both anterior d posterior horns can be inspected. An incision rallel to this, but higher up on the median aspect of e cerebral hemisphere, should then be made. If this made deep enough, the cerebrum can be pushed out- ard on either side and the lateral ventricles will

remain exposed to view. The corpus callosum should be divided at its anterior end and turned backwards. The basal ganglia can then be examined by making a series of parallel incisions transversely across the floors of both ventricles. The incisions should be a quarter of an inch apart and should extend almost, but not quite, to the basal surface of the brain. Split the cerebellum in the mid-line and, opening out the whole brain, examine the third and fourth ventricles. The veins and the choroid plexus, can now be investigated. Remove the cerebellum and examine the medulla and brain stem by a series of transverse cuts at intervals of one quarter inch. Examine the grey and white matter of the cerebrum by a similar series of antero-posterior cuts. The spinal cord is examined by a series of partial transverse sections.

The muscular and bony systems of the body are next examined, when necessary. In cases of anaemia, etc., a femur should be removed and the marrow cavity exposed by sawing the bone longitudinally. The femur is removed as follows: make an incision, starting at the inner side of the knee joint and passing transversely across to the outer side at the level of the lower margin of the patella. From a point just above the great trochanter of the femur cut through the skin and soft tissue on the outer side of the thigh. This incision should extend to the shaft of the femur and at the lower end, meet the transverse incision. Now flex the knee and expose the knee joint. Cut the ligaments of the knee joint and reflect all soft tissues from the femur. The femur will now be free, except at the hip joint. Insert the point of a knife through the capsule of the hip joint. Holding the point of the knife against the neck of the femur with the left hand, rotate the femur until the capsule is completely incised. On separation of the ligamentum teres the femur is completely detached. Dissection of the orbit, internal ear, and nasal sinuses may be required, and must be carried out through the skull base.

PART III

LABORATORY ANIMALS

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LABORATORY ANIMALS

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INTRODUCTION

121. Laboratory animals play an important role in boratory methods and diagnosis of disease processes. hatever be the size and status of a pathological boratory and whether it functions as a static unit peace time or as a field laboratory during war, it ust have "Laboratory animals" as part of its integ- l equipment, if it is to perform its role properly and lly. It is, therefore, essential that every pathologist ould be fully conversant with the facts pertaining the housing, breeding and proper care of the labo- tory animals.

The two points which vitally concern the user f laboratory animals are a regular and sufficient upply, and a constancy of quality. The quality and uantity of the supply will be closely bound up with ne satisfactory housing of the animals or, in other ords, comfort and cleanliness. The fundamental fac- ors in the comfort of the animals are temperature and entilation; their cleanliness is the key to prevention f disease. Of equal importance is the supply of a vell balanced diet and personal supervision and in- erest in their care. A clean colony, happy and well ared for, will mate better, produce hardier animals nd show more uniform experimental behaviour than vill a dirty, overcrowded and casually treated one. The provision of hygienic and comfortable quarters is hus not only aesthetically satisfying but economically ound.

The convenience of having stock diets pelleted or ubed is becoming gradually recognised as being la- our saving and economical. Instead of putting food

on the floor of the cage where it gets soiled with excreta, saw dust, etc, it is better to use wire mesh or bars basket, through which the animals nibble. The food is so arranged as to make the eating sufficiently difficult to ensure that any partly eaten cube which falls out is finished up before a further attack is made on the basket.

Apart from Vitamin C, the nutritional requirements of rabbits and guinea-pigs are similar. In guinea pigs, the serum complement does not reach maximum activity until the ascorbic acid of blood serum is at a level of 1 mg/100 ml of blood. This is obtained by a daily intake of 10 mgm of ascorbic acid.

Common salt (Nacl) is as essential for animals as it is for human beings. A large crystal of rock salt left in the cage or in the run supplies the required quantity. The animals lick at the crystal periodically.

Animals used for laboratory investigations for practical purposes are classified as under:—

(a) *Breeding stock*—Special methods are required for each type of animals.

(b) *Reserve stock*—Readily available for investigation purposes and should, as far as possible, be selected males only.

(c) *Experimental animals*—There should be a central register for each species of animals, like guinea pigs, rabbits, etc. and any animal taken by any worker should get a serial number from that register, where brief details of the experiment are also noted. eg. 4561—64. Inoculated with X organism from Kanpur. 4565—66 Bled for complement.

This serial number should be used for all sectional rds, where a complete record showing sex, colour, king, weight, etc, and experimental details are d.

Identification of animals—A dependable system entification of the animals should be adopted. The tice of cage labelling is suitable for stock animals in large groups. In the interest of complete re- s each animal should be numbered and registered regard to sex, description, etc.—

(a) *Tagging*—For rabbits and guinea pigs small aluminium tags may be used. They are held in place by small staples passed through the eyelets of the tag and then through the ear and the ends bent over. These are liable to come off or cause sloughing of the ear.

(b) *Banding*—A band bearing the number is fastened around the leg. There are several types of "leg bands" and varying the size of the band, it can be used for all laboratory animals including birds for which it is best suited.

(c) *Description*—If this method of identification is employed alone, animals of different marking or colour should always be selected when more than one are placed in a cage. Rubber stamps for drawing mouse, rat, guinea pig and rabbit may facilitate in marking these records. This method is very reliable and can be used in conjunction with the tagging to ensure proper identification, should the tag be lost.

The sex should be recorded as a part of the description (male ♂ and female ♀).

(d) *Markings*—Small animals like mice and rate may be marked by colouring the hair and

skin on the body or along the tail. The colo
ing is liable to fade and if the observation
to continue for more than a week, it sho
be re-applied. Cages should be labelled.

(e) *Tattooing*—This is a simple and efficient m
thod of identification and can be used for
laboratory animals. Tattoo needles are ea
procurable and cheap. A number can be
tooed on the inner surface of the ear.
other simple method is to scratch into
ear by means of triangular surgical nee
and rub well into it Indian or Chinese
and allow to dry.

OUSING OF COMMON LABORATORY ANIMALS

122. General principles applicable to all animal ouses are:—

Material of construction—Brick or stone is without oubt the best material of construction both for per-anency and for minimising changes due to weather. alls must be plastered and should have a coating of ashable oil paint. All angles and corners should be ounded and coved, and floors should have a slight ope to a drain to carry off wash water. A single on gully traplet flush with the surface in the centre f the room is better than a channel running through ie length of the room. The animal rooms must ob-iously be made rodent proof.

Design—The most important features of design re avoidance of overcrowding and provision of sepa-ate rooms for different purposes and operational eeds. These may be classified as breeding, testing or xperimental, cleaning and storage, etc. Width of angway between cage racks is important. This should ot be less than 3 feet.

Light, Heat and Ventilation—Rooms should be vell-lighted and ventilated, but large windows should e avoided as these increase heat loss and add to the difficulty of temperature control both in winter wind draughts) and summer sun. Windows and glass panels n the roof may be added to small windows high up n the walls.

It is an established fact that growth is retarded in animals kept in hot rooms and is accompanied by adaptation in heat loss organs as, for example, larger tail in rats and mice, and larger ears in rabbits. Food consumption is reduced, the onset of sexual functions is delayed and there is loss of fertility. Resistance to infection is diminished resulting in a considerable increase in the incidence of colds and pneumonia. It is,

therefore, obvious that in the greater part of India during summer, cooling arrangement with Khas Khas Tattis or Chicks with a continuous flow of water must be insisted upon. The ideal arrangement would be to instal a small air conditioning unit so as to maintain the optimum temperature of 70—75°F throughout the year.

The cages should be kept on racks or tables neither touching the wall nor each other. Shelves on walls are not recommended, one should be able to get all round the rack for cleaning.

Food and if possible bedding for the cages should be stored in containers to which wild rodents cannot have access.

A fresh consignment of animals should be quarantined for 3 weeks before adding to the general stock. In case of an outbreak, the room if possible, otherwise the animals, should be isolated and the rest of the stock re-distributed into the smallest possible numbers per cage. If more than one die from the cage the rest should be destroyed, and the same may be necessary for the whole colony in case of an extensive outbreak. For, the survivors are only worth saving if one is sure that they have not become carriers. An animal once brought out from the stock to the laboratory for experiment should never be returned if not used.

123. **Animal Cages**

Whatever the design of the cages the construction should be such that they are comfortable, easily cleaned, and easily disinfected and fairly durable to last for some years. A common feature to all the cages recommended below is that they are all-metal and except for rats and mice they have a wire mesh floor with 2" to 3" deep draw tray below to receive the excreta. Wooden cages of any design are not recommended. Metal cages galvanised after manufacture do not rust.

Washing of the cages, food containers and water es should be done in a separate room and at once a week. Steam sterilization of the cages referable but if chemical disinfectants are used act should be for a sufficient period, and these ld not be used again before they are dry. The rent types of bedding used in the cages are steri- wood shavings, sand, dry hay and cotton. The ns should not be left on the floor in a cage, but g on the side with a hook within reach of the nals. The water is best given through water- les. If prepared cubes of food, as recommended mice and rats, are used they can be left in a wire ket attached to the side of the cage with hooks. wire food basket for mice should be $\frac{1}{2}$" square h and for rats $\frac{3}{4}$" square mesh.

Two types of cages are recommended for each nal, a large stock and breeding cage and a small for experimental animals, meant for a single or ew animals. Guinea pigs require only an experi- ntal cage for they are stocked and bred in runs. mice, the same cage is used for all purposes or experimental animals can be kept in the same e as recommended for rats.

The measurements of different cages in inches are under:—

Animal	Type of cage	Length	Breadth	Height
bit ..	S & B	72	40	50
bit ..	individual chamber ..	24	40	15
bit ..	EC	24	18	18
bit ..	breeding chamber ..	19	14	13
ea pig ..	EC	12	18	13
.. ..	S & B for 16	18	15	18
	EC	10	10	10
.. ..	Universal	18	8	6

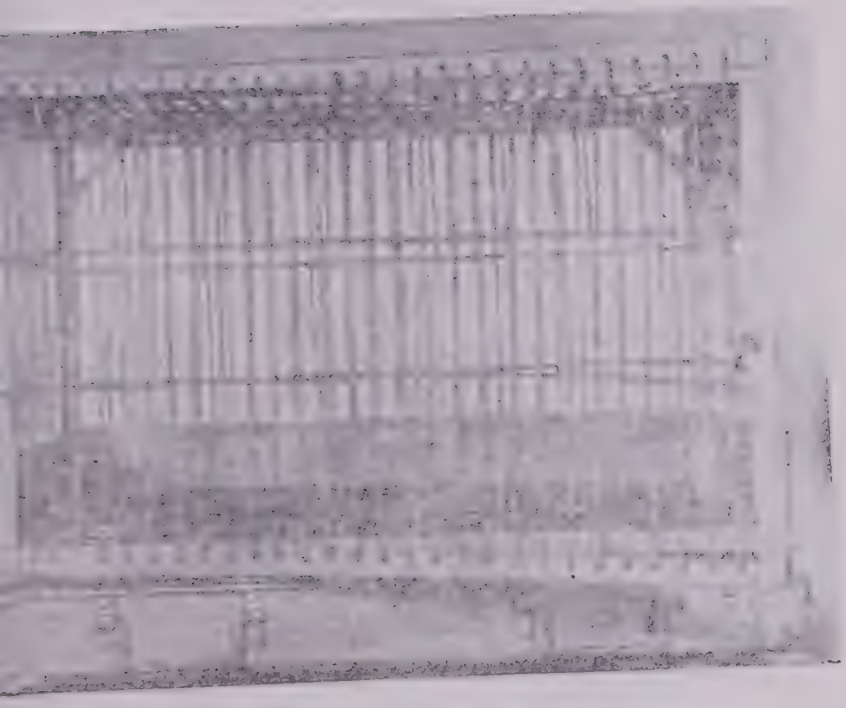
[illegible]—S & B—stock and breeding cage; EC—experimental animal cage.

The designs of the cages and measurements a
given in the following photographs and sketches.

FIG. No. 1

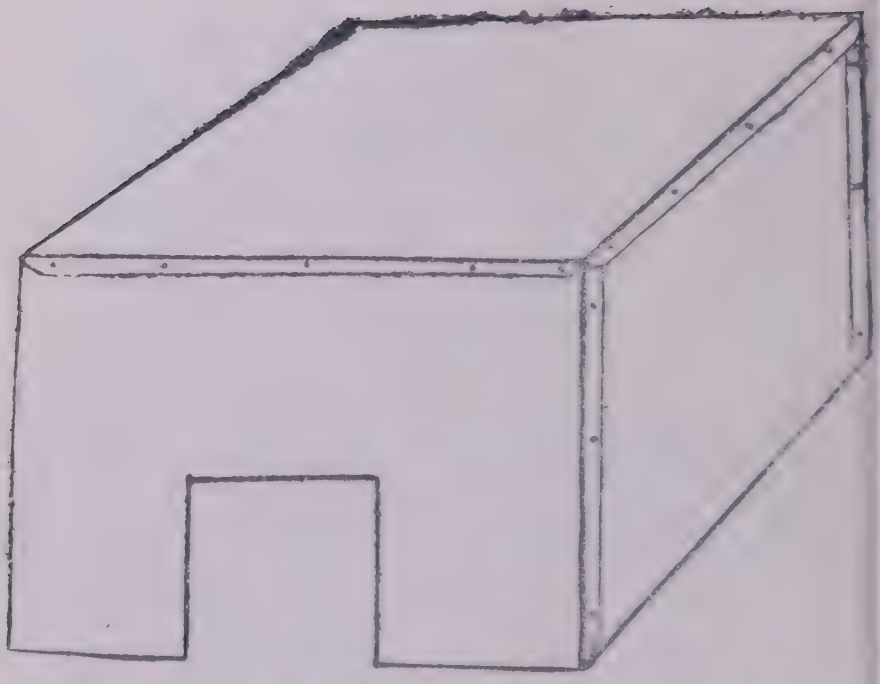
Rabbit :—Stock and breeding cage, preferably on rollers.
Total measurements :—72″ × 40″ × 50″
Individual chamber :—24″ × 40″ × 15″

FIG. No. 2



Rabbit :—Cage for individual rabbit.
Measurements :—24″ × 18″ × 18″

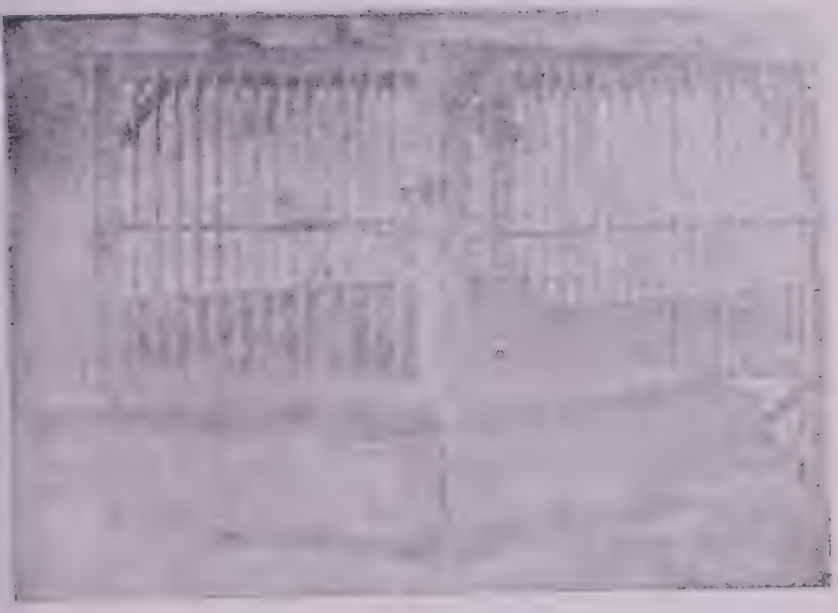
FIG. No. 3



Breeding chamber for rabbits :—This has no bottom, and is placed inside a chamber of the stock rabbit cage when necessary, for the doe to build a nest for its young ones.

Measurements :—19″ × 14″ × 13″

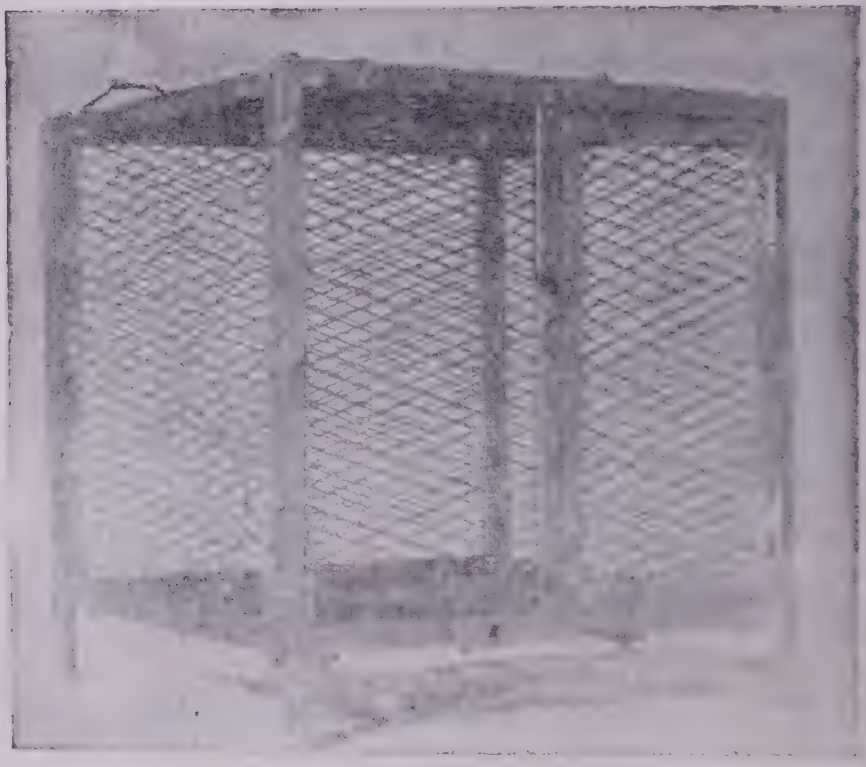
FIG. No. 4



Guinea Pig :—For keeping a single animal under experiment for any period.

Measurements :—12″ × 18″ × 13″

FIG. NO. 5



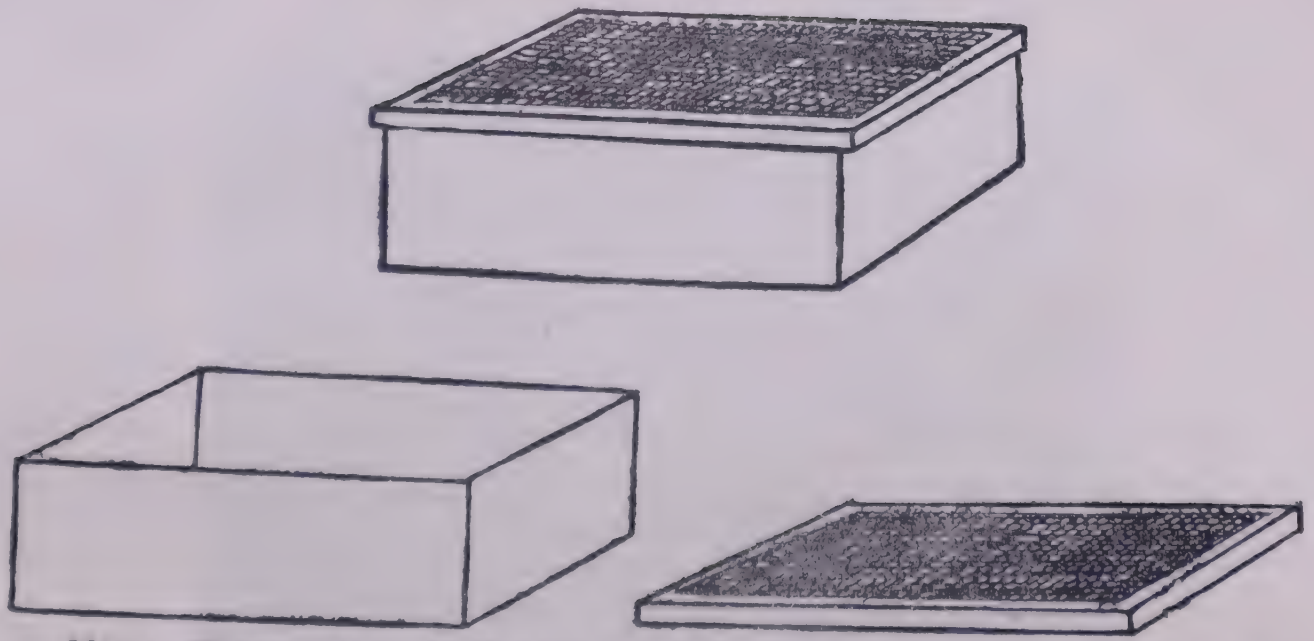
Rats :—Cage for keeping a stock of 16 rats for breeding.
Measurements :—18″ × 15″ × 18″

FIG. No. 6

uts :—For individual rats, can also be used for mice. The metal loops on the side are for the water bottle.

Measurements :—10″ × 10″ × 10″

FIG. No. 7



Mice :--The cage is made of stout metal sheeting at the bottom and on all sides. The lid is made of wire gauze held in a metal frame. It is not fixed to the box but just rests on it. The water bottle can be a test tube corked at open end and a hole blown on the side, and held on the inside of the lid with a wire loop.

Measurements:—18″ × 8″ × 6″.

GUINEA-PIGS

124. Guinea pigs are the most useful of all laboratory animals for routine and research work. To the bacteriologist they are "bacteriological barometers". They are handy animals, easily bred and clean in their habits. The two varieties of guinea pigs in use are:—

(a) Short haired, Angora.

(b) Long haired, Abyssinian.

The latter are larger animals and suitable for breeding for complement. For routine laboratory work the short haired (Angora) is preferable. They vary in size from 200 to 800 gms. Where over-crowding is prevented and good hygienic environments prevail, little trouble should be experienced in their rearing and breeding.

Uses in the Laboratory

125. Guinea pigs are useful for most bacteriological work of a diagnostic or experimental nature. The large size boars are used for cardiac bleeding and the white coloured for skin tests. They are not suitable for the raising of immune sera, eg. high titre agglutinating sera. They are used in the diagnosis of tuberculosis, typhus fever, Weil's disease, leptospirosis, etc. They are also used for the standardisation of antitoxins, e.g., diphtheria, tetanus, etc., and for the study of the anaphylactic reaction. Wasserman laboratories obtain their complement by heart puncture of healthy male pigs.

Housing and Breeding

126. Given plenty of fresh air, occasional sunshine and protection from draughts and wet, guinea

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pigs thrive well. Though primarily a sub-tropical animal it adapts itself to coldest climates but temperature of 112°F and above are usually fatal—

(a) If space is available a satisfactory method is to accommodate about 100 guinea pigs in large wire-netting pens. Cement concrete sloping floors drain off urine and facilitate washing. Loose hay bedding should be liberally spread over the floor during all weathers. It is cooling during summer and gives sufficient warmth during the winter, besides giving the animals cover and protection.

(b) Where the space is limited, housing in large angle iron cages with small wire mesh-netting and sectional trays to collect urine and droppings are ideal. A cage about the size of 6′ × 4′ × 1′ can accommodate 50 adults. These cages are ideal from hygienic point of view and can be placed in tiers of four, one over the other, and can be removed for cleaning and placing in the sun without inconvenience.

It is important that the temperature of the animal house is kept as even as possible. Guinea pigs are very susceptible to changes of temperature and deaths from this cause are not infrequent among the young.

When the type of housing described above is employed, each sectional cage must bear a registered number and label to indicate:—

(i) Breeding stock.
(ii) Growing stock (males and females separate).
(iii) Fresh stock for investigation.
(iv) Stock under investigation.

Breeding stock—Healthy and fresh, grown adults are selected for breeding stock. The condition of the coat is a good indication of health. They must be free

n any skin infection and vermin. A starry coat is cative of poor health and if in doubt take the al temperature.

Temperature of 103°F should be considered with picion.

Temperatures above 103°F are pathologic.

One male is placed per every 5 females. It is im- tant that the males should be changed to infuse blood and avoid incest. The period of gestation 66—72 days. From the time of birth the young ctically look after themselves. In a flock, they asionally lose contact with their mother but attach mselves to any female in lactation, as guinea pigs ke excellent foster mothers. At the end of 12—14 s, they are weaned and begin to nibble at food greens served to their mother. From then till ut 8 weeks, they require very little attention. They uld be sexed and caged separately at the end of eeks.

The female can produce three litters of young in ear. The usual number in a litter is two but limits 1—6.

Growing stock—Both males and females should nursed separately and liberally fed. If any of them undersized, weaklings or show signs of illness y should be destroyed. They are usually due to reeding or incest. They are not fit for experimental rposes until they are about 6 months old. The orous and those of good size should be marked for d and should be given individual care.

Stock for investigation—Male guinea pigs are pre- able for experimental purposes. They should be used separately and maintained in good health. ery precaution must be taken to prevent used ani- ls getting back into the flock. Apart from the possi- ity of spreading infections, they will certainly in- fere with investigations.

Stock under investigation—Same as other laboratory animals. There must be dependable system of identification. Every animal under test must be caged separately. Their registered number, weight, temperature, sex and "inoculated under investigation" must be recorded in the animal register.

Feeding

127. Guinea pigs are required to be fed twice daily. Food and water should be served in hook-on tins and not left in trays on the floor—a practice which aids spread of disease.

A morning feed consisting of the following is considered adequate—

Oats	1 part	About one oz per head.
Bran	3 parts	

Add water to moisten. An afternoon feed of about 2 ozs of green vegetables is all that a guinea pig requires.

Twice weekly, crushed grain may replace oats. Cod liver oil mixed with gram is an excellent conditioner particularly in wet weather. Water should be changed twice daily and should be always available.

Common Diseases

128. Para-typhoid and respiratory diseases caused by pneumococci, pasteurella or Friedlander bacilli are usually responsible for severe epizootics among guinea pigs.

Para-typhoid—Generally caused by Bact aertrycke and/or Bact enteritidis often found in healthy animals. The symptoms generally occur when the animal's natural resistance is lowered. It is liable to spread rapidly and a severe outbreak will follow if prompt measures are not taken to combat it.

Symptoms are not particularly characteristic and vary in different outbreaks.

As a rule the sick animals crowd together and are itated. Diarrhoea may or may not be present. disease may be confused with pseudo-tuberculo- ue to pasteurella and diagnosis is only established acteriological methods. Mortality rate varies from 70 per cent. At postmortem, the spleen is en- ed and small yellowish nodules are seen in the en and liver.

Pleural effusion with patchy pneumonic consoli- on of lungs are also seen.

Once the disease is detected it is wise to destroy sick animals and isolate the contacts. The vacated n, cages or pens must be thoroughly scrubbed and nfected. The contacts should be kept in small ps so that even if the disease spreads amongst n the number of contacts exposed to infection are ll.

Pneumonia—Caused by pneumococci, streptococci Friedlander's bacilli. The mortality is high among stock animals. The condition is usually diagnosed utopsy when the pneumonia is found to be asso- ed with a gelatinous blood stained exudate sur- nding the lungs. The bronchi are filled with a k sticky exudate. The lungs may be adherent to ura and there may be evidence of pericarditis. t. Friedlander can be easily isolated by routine ural methods.

Pseudo tuberculosis—It is characterised by the nation of caseous necrotic areas in the spleen, r and lymph glands. The acute form is septicae- with death occurring in 24 hours and no symp- s or lesions. Diagnosis is established by isolation he organism. In the chronic type duration is 4—6 ks and the disease is not usually diagnosed during At post-mortem, the necrotic lesions are found spleen, liver and lymph glands especially the

mesenteric. The disease is important in that it r sembles tuberculosis. Microscopically, acid fast bacil are not seen and bipolar staining pasteurella org nisms are present. Histologically giant cells are abse and epithelial cells are rare.

Pasteurellosis—Pasteurella septicaemia may occu among guinea pigs, though it is commonest in rabbi and mice. The sick animals have ruffled fur, ha closed eyes and they refuse feeds. At postmorte there is an exudate in the serous cavities especiall pleura. The lungs, liver and other organs may be co gested. At times there is little to be seen at pos mortem. Cultures of the exudate, blood, liver an spleen will establish the diagnosis. The bipolar stai ed organisms are well seen in the smears made fro the heart, blood or pleural exudate.

Other diseases—Streptococci also are responsib for epizootics among guinea pigs—Epizootic lymph denitis (lumps) and epizootic abortion are caused b haemolytic streptococci. Diagnosis is made at postmo tem by isolating the organisms. Coccidiosis is also e countered though not as frequently, as in rabbits.

Myco-tuberculosis—It is important to remembe that guinea pigs are liable to develop myco-tuberc losis infection from contact with cage mates that hav suffered from the infection after inoculation f diagnostic or experimental purposes with tuberculos material. It is, therefore, important to keep health guinea pigs separately from used or inoculated one which should preferably be destroyed.

Cervical adenitis—Usually unilateral due to strept bacillus is quite common. The infection occurs throug abrasion of the buccal mucosa from ingestion of r contaminated grain. The disease has hardly any sy temic effect, the prevention is obvious.

Coccidiosis—Infection with Eimeria cavae is wid spread. Heavy infection without clinical symptoms

te common. Symptoms when present are diarrhoea, cus in the faeces, loss of appetite and polyuria.

Paralysis—It is a virus infection. The affected ani- ls develop fever, become thin especially about ir hind quarters and paralysis of the hind limbs ows. The salivary gland virus causes no symptoms natural infection, but intracerebral inoculation ses death in young guinea pigs.

Piroplasm bigeminum—which is normally a di- se of cattle in India and conveyed by ticks was e observed in a guinea pig at Jubbulpore. It sed rapid emaciation and was fatal. Dry hay was ng used as bedding and it is possible that some was transported along with it.

RABBITS

129. Various types of rabbits are available but for laboratory purposes, the choice should be for the larger breeds, such as the Beverens, Chinchillas, Flemish giants, Belgian Hare, and the old English rabbit commonly known as the "Butterfly smut" from their nose markings. These breeds produce a better yield of blood and serum than common Indian rabbits. Many of these breeds are available in India. The Angora type of rabbits with their long fluffy coat should be avoided as they are difficult to keep clean. They pick up vermin readily and do not thrive in all climates though from commercial point of view, this breed is popular in the continent for its valuable wool.

Rabbits are often attacked by different parasitic infections and it is therefore important to breed own stock from healthy animals.

Uses in the Laboratory

130. Rabbits are used for diagnostic and experimental purposes. They are extensively employed for—

(a) Production of immune sera, such as agglutinating and haemolytic sera which are constantly used in routine laboratory diagnosis and complement fixation tests respectively.
(b) Standardisation of diphtheria antitoxin.
(c) The preparation of media, eg. NNN media.
(d) Study of staphylococcal toxins.
(e) Friedman's test for pregnancy.
(f) Differentiation of bovine from human type of tuberculosis.
(g) Study of certain virus and spirochaetal diseases like smallpox and syphilis.

Housing and Breeding

131. *Breeding*—For breeding rabbits, the stock should be selected from adults 5 to 6 months of age and in bloom. They must be free from vent trouble and must not show the least sign of cold or loose stools.

The stud buck should be housed separately and well fed. A record of his age should be maintained, and when over three years he should be discarded. Old buck should be mated to young doe and vice versa. Young, born from parents over three years usually show signs of degeneracy and those from parents under ten months are normally, if not always, rickety. Mating should never be allowed, unless both buck and doe are in bloom and in hard coat. Mating should be avoided during moulting.

There are two systems of housing during breeding—

(a) HUTCH system.

(b) WARRAN system.

Hutch system—When the doe is ready for mating she should be placed with the buck. Bucks should not be placed in the doe's hutch as the doe resents any intrusion into her privacy. If she is ready, she will intimate her desire by stamping the floor with her high legs. Mating should never be forced. If the doe is not in season she should be removed from the hutch and replaced daily until serviced. Two services or "fall" are quite sufficient.

The date of mating must be registered and for ready reference may be chalked on the hutch. if after about the 10th day she is seen to pluck her fur she has probably missed and she should be placed with the buck again. If she refuses the buck, she should be removed. She should not be left for a long period without supervision as the buck in his anger may pull her about and even kill her. A shy unwilling doe

should be kept in a hutch which previously contained a buck. This helps to bring her into season. After successful mating she should be placed in her breeding hutch and should be left alone. A doe likes seclusion when she is in kindle or pregnant.

When about to kindle, the doe can be seen plucking her fur from around her nipples. A liberal supply of coarse saw dust and a little hay is placed. Does like building their nest out of straw and they can be seen carrying hay about in their mouth.

Breeding hutches are best made to accommodate a single doe so that they can be moved without disturbing one another. The period of gestation is 30-31 days. During this period the doe must be well fed with increased ration and water. Milk if available should be given during the last week. A warm mash consisting of bran, boiled vegetables, used tea leaves with crushed oil-cake and a teaspoonful of cod liver oil may be given as an additional meal.

This feeding must be maintained during the period of lactation also.

Rearing—A litter may be of 2—18. the average is 8. When the young are born they should be left alone for 48 hours. Take the doe out without exciting her. Rub the hands well in the saw dust of the hutch before handling the young ones. Does resent interference and through nervousness devour the entire litter or neglect them. It is best to remove this type of doe from the breeding stock. Without disturbing the nest, gently remove the deformed and the weaklings. About 7-8 may be left to the doe, the less the better, as they obtain more nourishment from their mother and grow faster and stronger. Get the doe as soon as possible back in the hutch, with a good supply of greens to get her to eat for a while. Weaning is at 6 weeks. When 10 weeks of age, they should be sexed and separated. If in-breeding is desired it is wise to ring the stock with numbered ring bands for

ecord of strain; name your breeding stock and stars "pedigree tree".

Stale food rapidly causes illness in the young and nust be removed from the hutch. Young rabbits rom different nests should not be handled at the ame time. As soon as the young are able to run bout, the whole hutch can be washed, disinfected nd fresh saw dust and hay placed. When about seven veeks old the young should be weaned and the doe should be removed to a separate hutch and left alone o recoup. The young should remain in their hutch till about ten weeks of age, when they should be sex-ed and separated.

The Warran system—This is a free system of housing buck and does together, usually ten does to a buck. By this system nests are arranged around the walls in cubicles, leaving a central hole of about six inches as entrance. The cubicle may be constructed of bricks with removable tops of wooden planks for easy cleaning. The floor should be made of concrete to prevent the doe from furrowing. This method of collective housing is not satisfactory as it is difficult to make it rat proof and there is danger of rabbits fighting and getting hurt when carrying young. The young are invariably overworked. Besides, the buck may destroy the young.

Feeding young—The same type of food may be given but they must not be allowed to gorge themselves. Bread and milk mixed with a little cod liver oil is good.

Gram must be well ground and moistened, before serving. They should get sunshine daily for a while and returned to their hutches as it gets warm.

Feeding

132. *Feeding*—Careful feeding is important in successful rabbit keeping and the condition of rabbits depends to a very large degree upon the quality and quantity of food supplied.

As a general rule, feeding twice a day meets their requirements. The feeding time depends on climatic conditions. 0900 hrs and 1700 hrs are usually suitable. The foundation feed should be crushed oats given dry. A suitable change is one part of wheat mixed with three parts of crushed oats. Bran may be given as a substitute for oats. Crushed grain is also used. A weekly feed of small quantity of oil-cake of cod liver oil mixed with meals is an excellent pick-me-up. When oats or wheat are not available mixed grain should be used.

Green foods—Rabbits eat almost every type of herb and vegetable. These should be given as a second meal to make up the diet. Lucerne grass is the one of choice as it is readily available, nourishing and relished by all laboratory animals. It must be fresh and green. Stale lucerne must be avoided. Lucerne must be spread out to allow free circulation of air, until such time as it is used. For storage, spread out on a tray with wire mesh bottom.

Harial (doop) grass is an excellent substitute for lucerne. Among vegetables cauliflower leaves, carrots and turnips are the best. Raw potatoes, pumpkins' marrow and beans should be avoided.

Water—It has been shown by some observers that rabbits can be reared without water. This may be possible in countries, where green vegetables are available all the year round. In hot climate, water should not be denied. They need water and take it well.

Butter milk—Is an excellent food, can be mixed with cereals or brans or given as such.

Tea leaves—Used tea leaves mixed with other meals are relished. It tightens up their coats and gives them a healthy look.

Food receptacles—Food should not be placed on the floor. Tins should be suspended by hooks to cage

hutch and should be of such a size as to contain st sufficient food for one animal. The rabbits have habit of picking up these tins and scattering food d water. Special attention should be paid when re- acing tins. Green food should be placed in "Wire cks".

Quantity—The following daily ration is adequate r a full sized rabbit:—

rning	Oats Bran	1 part 3 parts	3 ozs.
ening	Lucerne		8 ozs.
ater during the day ..			4 ozs.

Crushed gram or crushed Indian corn may replace ats. Gram should be soaked overnight before serving. ood should not be stored in large quantities as it gets ouldy particularly in wet weather and should be tored in rat proof bins.

ommon Diseases

133. *Coccidiosis*—Three different species infest the abbits. E, steidae enters the blood stream from the ntestine and lodges in the columnar cells of the bile ucts of the liver. The oocysts enter the gall bladder nd from there the intestine and the faeces. Another pecies E. perforans parasitises the intestinal epithe- ium, causing rupture of the cells and haemorrhages n severe infestation. A massive infection in sucklings an be rapidly fatal. The symptoms are ravenous ppetite and emaciation. E. magna is a parasite of the mall intestine and pathogenic.

The infection can be cured and controlled by ad- ninistration of Sulphamezathine in drinking water or ood.

Mucoid enteritis—A disease of unknown etiology with a high death rate of 100 per cent. in the sucklings o 33 per cent. in the young. The prominent symptom s emaciation of the muscles of the back and the hind quarters. There is no fever and the onset may be sud- den. The only postmortem signs may be in the kidney

in those dying after weaning age. Due to the cause being unknown, no particular control measures can be applied.

Snuffles—The disease is attributed to Pasteurella lepisepticus and Brucella bronchiseptica. The mild form does not affect the health. The symptoms are bouts of sneezing with or without nasal discharge. In the chronic form the nasal discharge becomes marked, the animal becomes weaker and dies of terminal pneumonia. In the acute form the primary nasal discharge is quickly followed by fever and rapid death. The best control measure is eradication of the affected and the contacts.

Pseudo-tuberculosis—It is caused by Pasteurella pseudo-tuberculosis rodentium conveyed by ingestion of material soiled by the excreta of infected mice and rats or of other rabbits. It is a chronic disease producing progressive emaciation. Postmortem there are primary caseous nodules in the intestine particularly caecum and secondaries in the mesenteric lymph glands, spleen and liver.

Rabbits can also suffer from Bovine and Avian type of tuberculosis, though rarely.

Pyogenic infection due to staphylococci, fusiformis necrophorus, etc., may occur and septicaemia due to quite a few organisms has been reported. Domesticated rabbits can suffer from syphilis due to Treponema cuniculi.

Diarrhoea in the young may be due to error in diet and may prove fatal. Loose stools should receive immediate attention. The anus of every rabbit should be examined and those with soiled anus or tail isolated. Green food should be withheld and dry food given. Five drops of tincture opii in a tea spoonful of olive oil may do good. Some of the other infections are labial necrosis and virus III.

Ear Canker—It is due to two species of mites Psoptes communis and Chorioptes cuniculi, which produce scabs inside the ear. The scabs should be softened with a vegetable oil, removed with forceps, and one of the mange dressings "Odylene" or "Folliculine" applied to the affected part. Body mange is due to Sarcoptes cuniculiand Notoedres cuniculi. The fur falls off from parts of the face, head and roots of the ears. The skin gets covered with yellowish or greyish scaly crusts. There is intense itching and scratching and soreness. The rabbit should be isolated to prevent further spread. Remove the scales and clean the part. Application of benzyl benzoate and Gammexane will almost clear the condition.

Rabbits can also suffer from the larval stages of the two dog tape worms. Taenia pisiformis and T. serialis. There are a large number of other diseases from which rabbits have been reported to suffer—like Rabbit pox, Toxoplasmosis, etc.

WHITE MICE

134. They are usually employed—

(a) For the isolation of pneumococcus in pure cul ture and for its typing.

(b) Extensively in typhus research, as it serves t demonstrate the typical pathognomonic peri toneal reaction in Scrub typhus.

(c) For the preparation of antigen against murin strains of typhus.

(d) Isolation and titration of lansing type of poli virus.

They are handy animals. They thrive and multiply well, provided they are given rich well balanced die and placed in suitable cages in a room free from draught. An even temperature must be maintained as white mice are very susceptible to changes of tempera ture. In the hot weather they should be protected from hot winds. They like cages that are not too well light ed.

Housing and Breeding

135. White mice are shy animals and are best housed in a special room. In view of the disgusting odour normally present, the room should be well ventilated and yet free from draught. All entrances should be rat-proofed. During hot weather a simple and yet effective method to maintain an even tempe rature is to fix perforated half-inch piping over the main door controlled by a tap. The door should be provided with good thick khus khus "CHICKS" to cover the entire door. By regulating the flow of water a continuous drip can be maintained. This will lower the room temperature considerably.

Each cage must bear a registered number for purpose of identification. A full description including sex, inoculum and colour dye used for staining the animal must be entered in a special register.

A wet and dry bulb thermometer should be available in every centre. Given an even temperature and well balanced food, there should be no difficulty in breeding mice. To every four females place two males. After ten days remove the male, and do not use him again, until he has had a fortnight's rest. A male used for three consecutive periods should be replaced. Females should be rested after each litter, and replaced after three litters or when one year old.

The period of gestation is 21 days with an average of 6 in each litter. Four litters are normal in a year. The females in one cage will litter together. The cage should contain paper cutting from which the females prepare their nests. In all the animals which produce large litters, the young are born blind. In ten days their eyes open and they wean in about four weeks. The female can be remated four weeks after weaning the litter.

Feeding

136. The mouth of mice is adapted for dry food, seeds and nuts. Therefore it is essential that all food should be as dry as possible.

Two feeds daily are sufficient. An adult mouse requires about an ounce of food daily.

It is necessary that this small amount contains all the essentials of a balanced diet.

The following formula is recommended for 75 mice for a week:—

Wheat	300 gms
Jowar	90 „
Bajri	90 „
Tur dal	15 „

Masur dal	..	..	..	..	15 gms
Soya beans	..	..	..	..	30 „
Sodium chloride	..	..	..	..	15 „
Meat	..	..	..	..	25 „
Milk	..	..	..	..	20 ccs. per mouse.
Cod liver oil	..	..	..	..	20 ccs. mixed with 3 lbs. of milk.

The first six ingredients are powdered, mixed with salt and meat, and baked in the form of small cubes. Cod liver oil mixed with milk is given separately. A green leaf of lettuce or small lucerne should be allowed. Germinating seed such as grams or millet seeds are given twice weekly. Cubes of raw potatoes are relished. For young stock at time of weaning, milk and bread given as dry as possible is relished.

Ground nuts are an excellent change twice weekly. Water should always be available and best given in inverted bottles with a "U" glass tube.

Uses in Laboratory

137. Mice are very useful for the isolation and study of:—

(a) Scrub typhus.
(b) Relapsing fever.
(c) Virus infections especially yellow fever.
(d) Standardisation of insulin and anti-toxins like tetanus welchi and histolyticus.

They have been employed for studies in herd immunity and research on neoplasms.

Common Diseases of Mice

138. White mice can be infested with a large number of parasites which do not normally affect their health, but under experimental conditions may become serious or influence the result of the experiment. The following are the common parasites.

Endamoeba muris—Amoeba enterica; A. musculi and A. faecalis; Trichomonas muris; Hexamita muris and Giardia muris. Of these the last one in severe infections can cause enteritis. Trypanosoma duttoni thiroux occurs naturally in the blood of the mouse and though non-pathogenic can occasionally be fatal. Coccidiosis due to Eimera faliformis. A mild infection may not harm but an acute one may cause enteritis. If re-inection is prevented it may clear in 26 days.

Cryptosporidium muris; C. Parvum and Klossiella muris are the other coccidial infections.

Sarcocystis muris—It affects the muscles where it can be seen as tiny white streaks. In some cases there are no serious results. Death may occur with heavy infection. The prevention is by avoiding contamination of water and food by urine and faeces of mice and by keeping the room free of fleas and cockraoches. The cages can only be disinfected by steam bath, for cysts will not be killed by disinfectants.

Toxoplasma—The infection was once detected at Imphal in 1945 in a mouse colony of Swiss and Welcome strains imported from England. There were no apparent symptoms, but when an injection was given with typhus infected material intraperitoneally, the rickettsia failed to multiply and toxoplasma were seen in peritoneal smears.

Helminthic infections—Taenia teniaformis from cats and T. pisiformis from cats and dogs may infect mice. H. fraterna does not require an intermediate host. H. Diminuta is important for it can some times occur in man. The intermediate hosts are rat fleas: X. cheopis and N. fasciatus. The adult lives in the duodenum and common bile duct. Sometimes it penetrates the liver when, due to destruction of parenchymatous cells and focal necrosis large abscesses develop.

Infestations:

Lice—Polyplax serrata. The infested animals look unhealthy. Like fleas they can convey any organism

present in the blood of the host from one to another. A 10 per cent. DDT powder should eliminate them if applied to the fur. The mouse flea is Leptopsylla masculi Duges but rat fleas can also attack mice.

Mites—The tropical rat mite Liponyssus bacoti can infest them and is known to convey Murine typhus from one infected animal to another. Echinolaelaps echdinunus is a vector of Hepatozoon muris of the rat. Myobia musculi and Myocoptes musculinus are the other mites that cling to the base of their hair.

Bed bugs—Cimelectularius Linne can adapt itself to living on laboratory animals.

Bacterial and Viral infections—Mouse typhoid is due to Salmonella typhimurium and S. enteritidis but epidemics due to other organisms are also reported. The signs of illness are that the animal sits quietly in a corner of the cage. hunched over, with head bent down and the hair ruffled. There is loss of appetite and weight; conjunctivitis develops. The faeces are softer and lighter in colour and death occurs within a week.

The mice are also susceptible to pasteurellosis, pseudotuberculosis; mouse septicaemia or Erysipelothrix, Streptobacillus moniliformis and pleuropneumonia like organisms.

Pneumonic infections with Brucella bronchiseptica, Friedlander like bacillus. and an Influenza like bacillus also occur.

Spirillum minus infection in white mice has been observed in some colonies in India.

The virus diseases of the mice are infectious ectromelia. Lymphocytic choriomeningitis and Encephalomyelitis of mice.

WHITE RATS

Uses in the Laboratory

139. The white rat is a good substitute for the guinea pig in the investigation of Pasteurella pestis. In Murine typhus, it is used for the storage of strains for periods upto six months and is, therefore, ideal for field research works. Scrub typhus infection also survives in them for 2 to 3 months.

Housing and Breeding

140. They require no special cage or arrangement for the purpose of breeding. Adult rats 4 to 12 months of age are allowed to breed. Given some paper cutting, shreds of cloth or cotton wool they build their nests in a corner of the cage and breed prolifically. One male is allowed for 3 females. The period of gestation is 21 days. They produce 5 litters a year. A litter can be of 1—17, the average is 8. Care must be taken when handling a rat as he can easily inflict a severe wound with the long sharp incisors.

Feeding

141. Food is the same as that for mice except that the daily ration is two ounces per white rat.

Common Diseases

142. The most common disease of rats is Paratyphoid due to several species of Salmonella group, the most common being S. enteritidis and S. typhimurium. Some of the other infections are Bronchopneumonia due to Streptobacillus moniliformis and a pleuro-pneumonia like organism. Purulent lymphadenitis and polyarthritis due to a filtrable agent,

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middle ear disease leading to labyrinthitis may also occur. Mange attacks rats, the common sites are ear, tail and lower part of the back. Neoplasms that occur in other mammals may occur in rats, their frequency varies in different colonies.

Rats older than 15 months should be destroyed or otherwise disposed of.

SHEEP

143. Rams are kept in laboratories for collecting blood necessary for Wasserman reaction and other complement fixation tests. In small laboratories, blood may be used for preparing media.

144. They should be allowed to graze during the day and need locking up only during the night. Their wool should be clipped short once a year. Apart from feeding they require very little attention. They are apt to fight among themselves and suffer wounds which must be promptly dressed with turpentine and sulphanilamide. Such wounds are painful and if neglected may lead to emaciation and death.

145. Sheep suffer from infections due to salmonella and gas gangrene groups of organisms. The only common disease is myasis.

146. *Diet*

Bran	..	..	1 Part
Gram	..	..	3 Parts

Two pounds per day and served twice daily. One pound of lucerne or any green fodder per day is necessary.

ANAESTHESIA

147. The choice of an anaesthesia depends, on whether the operative procedure is of a short or prolonged duration. The terminal method followed will depend on the purpose for which the tissues are required such as passage of a strain, histo-pathology, or biochemical determinations. The mode of administration and the properties of some of the common anaesthetics are as follows.

Ether—It is generally given by the open method with a wire mask covered with gauze or lint and an appropriate size according to the animal. Too much ether is indicated by extremely rapid, shallow breathing or spasms which usually appear first in the hind legs and are synchronised rhythmically with breathing. Insufficient ether is indicated by respiratory spasm and struggling.

It is preferable for operations of short duration and as a terminal anaesthesia for careful histological work. It, however, causes a marked glycogen diminution and is, therefore, unsuitable for glycogen determination of liver. Similarly any violent terminal method like decapitation, exsanguination causes a considerable loss of muscle glycogen due to glycogenolysis produced by hypersecretion of epinephrine.

Chloroform—It is administered like ether, and can be used as a terminal anaesthetic, but is undesirable in operative procedures; for, deaths in a few minutes due to chloroform are rather common. Of course it can be safely combined with ether if an even and deep respiration is desired for a short period, particularly for intranasal administration to mice. In this case a mixture of one part of chloroform and two parts of either is used. Cotton soaked in this mixture is kept

at the bottom of a wide mouthed glass jar; over this some dry cotton is kept so that the mice do not directly come in contact with the anaesthetic. The mice are put in the jar covered with a petri dish and progress watched.

Evipan—The solution should be made fresh and given intraperitoneally. It is of low toxicity, there is quick recovery and it causes no respiratory complication. Young rats are intolerant to it. It is useful for short anaesthesia or narcosis.

Nembutal—The solution is made in water, preferably from accurately weighed powder and not ampules and should not be stored for more than 48 hours. Rabbits can be given this solution intravenously and rats intraperitoneally. If anaesthesia becomes too deep strychnine can be given 1/100 grain per 200 G. body weight. It is a fairly safe anaesthetic for a prolonged operation. Intraperitoneally the anaesthesia is complete in 5 to 15 minutes and lasts for nearly 45 minutes in rats.

Avertin—It is given intraperitoneally. This is another safe anaesthetic with a good latitude and has been used for guinea pigs and rats. Unconsciousness occurs in a minute and if an initial light anaesthesia is given with ether the animal passes from one to another without recovery. Unconsciousness is easily maintained for one hour and the respiration remains deep and regular.

148. **Anaesthetics for different Animals**

Rabbit—

(a) Open ether; and to depress secretions atropin 1/50 grain for an adult may be injected.

(b) Evipan sodium. 0.6 ml. of a 10 per cent solution with initial ether.

(c) Nembutal. 0.6 ml. to 0.75 ml. of a 6.5 per cent. solution intravenously, supplemented with initial ether.

(d) Terminal anaesthesia, or euthanasia.

(i) One ml. of saturated magnesium sulphate solution intravenously.

(ii) A sharp blow at the back of the neck; this requires practice.

(iii) Ether or chloroform.

Guinea pig—

(a) Ether or ether and chloroform mixture (ether two parts and chloroform one part)

(b) Urethane: One ml. of a 2.0 per cent solution given intraperitoneally.

(c) Avertin: For 250 to 400 G. guinea pig 0.8 mg. per 100 G. body weight of a 2.5 per cent. solution; for 400 G. and over 1.0 mg. per 100 G. body weight.

Rat—

(a) Ether and chloroform mixture.

(b) Avertin. 0.5 mg. per 100 G. body weight intraperitoneally.

(c) Nembutal. For light anaesthesia 3 mg. per 100 G. body weight.

For deep anaesthesia 4.5 mg. per 100 G. body weight.

(d) Evipan. For narcosis 5 mg. and for anaesthesia 10 mg. per 100 G. body weight.

Mice—

Ether and chloroform mixture.

MINOR OPERATIONS ON ANIMALS

149. In this section it is proposed to give a short description of various minor operations that have to be carried out by any one engaged in animal work. It is realised that skill and perfection in these operations can only come after experience and carrying them out several times, but it is hoped that if these methods are followed, proper skill will be achieved with less difficulty.

Certain cardinal rules must always be respected while conducting any operation on the animals. It must be remembered that in all animal operations the same care should be taken as in human operations. No intentional pain must be afflicted and anaesthetics should be employed without reservation. After the operation the animal should be left in the care of a trained person in a secluded dark place.

It is important to prepare the animal before operation. It should be starved for 12 hours prior to the operation. When it is necessary to remove hair from the area of operation it should be done either by clipping and close shave with a safety razor or by a depilatory mixture, e.g. equal parts of Barium sulphide and starch mixed in water or such proprietary products as "Depil". When depilation is done it should be carried out a day before the operation.

The instruments must be properly selected. The blunt instruments are always the cause of delay and accidents. Syringes must not leak and the needles used must be sharp. Sterility of all the instruments must be assured before any operation. It is essential to remember that a hesitant attempt always inflicts more pain and causes more deaths than a bold attempt.

150. **Methods of Inoculation**

(a) *Subcutaneous*—In smaller animals, eg., mice and rats, the subcutaneous injections are best given at

the back and in larger animals, e.g., guinea pigs and rabbits, on the ventral surface of the thigh. In each case the skin is pinched between the thumb and index finger of the left hand and the needle inserted at the base of the pinched skin. The skin is then allowed to fall back and the needle is moved when it can be seen or felt to be just under the skin. The plunger of the syringe is then withdrawn slightly to see that the needle has not entered any blood vessel and the material is slowly injected.

(b) *Intradermal*—For skin test intradermal injections are frequently given. They are best given in shaved abdominal skin. The skin is pinched up between the thumb and index finger of the left hand and then stretched and allowed to rest on middle finger. With a fine bore, short bevelled needle attached to the syringe, the skin is pierced as superficially as possible and the material injected. If successful, a small and very circumscribed wheal is raised which does not disappear when the skin is released.

(c) *Intraperitoneal*—The best site for intraperitoneal injection is slightly to the left or to the right of the midline of the abdomen at a point where the lower 1/3rd of this line joins the upper 2/3rd. The animal should be held head downwards by an assistant and the needle inserted at an angle. The sudden disappearance of resistance and the mobility of the needle indicates that the peritoneal cavity has been reached and then the material is injected. The needle is sharply withdrawn with a jerk. The danger of piercing the intestines is very much less, provided the animal is held head downwards for some time and the injection is given sufficiently low in the abdomen. A much more frequent mishap is to pierce a full bladder but this can be avoided if the site of injection is properly selected. Some operators prefer to give intraperitoneal injections from either flanks but, though reasonably safe in expert hands, this method is fraught with the danger

either piercing the kidneys or the ascending or descending colon which are fixed structures.

(d) *Intravenous*—Different veins are chosen in different animals.

(i) *Rabbits*—The lateral ear vein is the one of choice. Though the middle veins are larger and more prominent these are very mobile, difficult to enter and thrombose quickly. The lateral vein though appears slender admits much thicker needles. The vein can be made more prominent by flicking, warming or by application of xylol but this is rarely necessary. If xylol is used it must be removed with alcohol, otherwise there is severe irritation and inflammation of the ear. The use of the box, through which only the head appears outside, is strongly deprecated because the animal is frightened, gets excited and kicks violently. It is always preferable to get an assistant to hold the animal and also press the vein at the root of the ear. The ear is stretched out by left hand and the site of entrance is supported by the middle finger below the ear. The vein is entered gently and the needle can be seen inside the vein. As the material is injected the vein is seen to get freed from blood. If the vein has not been entered the material is not easily pushed in and slightest resistance to the plunger should indicate that the vein has been missed. The injection should be slow and material flows in easily if a slight but constant pressure is maintained on the plunger by the thumb.

(ii) *Guinea pigs*—The prominent superficial vein winding round the hook of the hind limb is the vein of choice. The leg should be shaved and stretched by the left hand. The vein is easy to enter.

(iii) *Rats and mice*—The lateral tail vein is on of choice. The veins are made prominen by immersing the tail in a beaker of h water of 50°C for 2 minutes. It is prefe able to anaesthetise rats before an handling, but mice can be steadied by a assistant by pinching the skin of the nec and stretching the tail. For both mice an rats a suitable container can be improvise by using a tin tubing of suitable diamete fitted with a cork from which a wedg shaped piece has been removed. Th animal is kept inside the tubing but th tail projects out through this cut. A fin bore needle is necessary and the entranc of the vein is determined by the ease i pushing the material in. This operatio requires considerable experience and th required skill can only be achieved b **practice.**

(e) *Intracerebral*—

(i) *Rabbits and Guinea pigs*—Shave the hea and sterilize the skin. Under anaesthesi the skull is exposed by an incision midwa between the eye and the medial ridge o the junction of the occipital and parieta bones. A small trephine opening is mad and injection carried out by means of 1 c syringe with ½" needle. An alternativ method is to hold the animal down firml after anaesthesia when a second assistan steadies the head. The skin is sterilize and stretched to one side and the hole mad by the drill which is controlled carefull so as not to let it go in suddenly. The injec tion is now completed. The skin is no allowed to come back to its normal positio and so covers the hole in the skull. Th wound is sealed with collodion. In case where no drills or trephines are availabl

the fine point of a pair of scissors or a sterile sharp drawing pin may be used for making the hole; in both cases the risk of suddenly going in is very slight.

(ii) *Rats and Mice*—A fine needle is used with a guard about 1 mm. from the tip. A slightly stouter needle is advisable for rats. Light anaesthesia is administered and the needle is introduced at a spot in the middle of a line drawn from eye to eye. With a slight boring movement the skull is easily pierced and the material is injected.

leeding of animals

151. Small and large quantities of blood have to e frequently drawn from animals either for other ests or to judge the results of experimentation. Different methods have to be applied in different anials.

(a) *Sheep*—The bleeding is done from the external jugular vein. The wool is clipped short or depilated over the neck. Animal is steadied by an assistant standing astride the animal and holding its jaws. A large bore needle is pushed into the vein and blood collected. About 30 to 40 ml. of blood can be removed at one bleeding.

(b) *Rabbits*—Small quantities of blood can be obtained from the lateral ear vein. All the hair is shaved off from the site and thinly smeared with vaseline to the edge of the ear. The vein is punctured by a sharp glass piece or opened longitudinally by a cutting needle. The proximal end of the vein is pressed between the thumb and index finger and drops of blood collected in a test tube. After the collection is over the bleeding is stopped by firm pressure over the puncture and the wound is sealed with Tr. Benzoin or collodion.

For larger quantities, the blood is collecte from the heart. The fur of the chest is clippe short and the animal is anaesthetized. The an mal is laid on its back and an assistant stand ing on the right of the animal holds the hin legs in his right hand and the forelegs en circling the head in his left and stretches th animal out. The operator stands on the sam side as the assistant, places his left thumb o the right of the rabbit's chest pushing th mediastinum over to the left side and fixing while the middle finger of the same hand fee for the apex beat. The syringe is held in th right hand like a pen and the needle is inser ed vertically between the ribs in the spac over the apex beat close to the sternum. Onc through the skin, short thrust should perfo rate the heart. When a proper syringe is use the force of blood is sufficient to push th plunger back thus showing that the heart ha been reached. The syringe is now passed t the left hand and plunger withdrawn ver gently with the right hand. In no case forc shou'd be applied on the plunger or the bloo withdrawn rapidly. After the desired amoun of blood has been withdrawn the needle removed sharply. The animal should be starv ed for 12 hours before the bleeding and give plently of water after the bleed. In commo Indian rabbits it is not wise to take mor than 20 ml. of blood at one time. If the tech nique described is followed and the heart en tered first time, death from cardiac punctur is rare. The syringe recommended is a Luer lok type of 50 cc. fitted with a 18—gaug needle.

(c) *Guinea-pigs*—

For a drop or so of blood the ear vein may b punctured. For larger amounts, cardiac punc ture is necessary. The procedure is same as i

case of rabbit. About 5 ml. of blood can be drawn at a time.

(d) *Mice and rats* —

Rarely if ever more than a few drops of blood are necessary. this can be obtained by a sharp incision over the tail vein.

(e) *Pigeons*—

Pigeon blood is used for preparing media or for haemagglutination test. Bleeding can be done from the wing vein or from the heart. The wing vein is exposed by plucking off a few feathers at the junction of the body and wing. The vein is a fairly large one and blood can be obtained either by a syringe or by a longitudinal cut of the vein. It is sometimes difficult to stop the bleeding and a firm pressure for some time may be necessary before sealing the wound with Tr. Benzoin or collodion. Cardiac puncture is a more difficult procedure. The bird is held by an assistant with breast uppermost and legs and wings held downward. The feathers over the breast bone are removed. The edges of the breast bone are felt with the left index finger and the notch on the left side is felt. This is normally about an inch above the tip of the breast bone. With the syringe in the right hand the needle is inserted close to the notch and keeping close to the breast bone the needle is passed in the direction of the right shoulder for an inch and a half. Frequently the beating of the heart can be felt transmitted by the needle. Gently withdraw the needle at the same time exerting slight suction on the syringe. When the blood appears, cease withdrawing the needle and draw about 5 ml. of blood. No anaesthesia is necessary.

In conclusion it must be emphasized that gentle handling is always a prerequisite for all

minor operations. The animal should be allowed a firm foothold while anaesthetic is administered, otherwise on a smooth table it slips and gets nervous and excited. Operations are best carried out in the morning hours and the animal observed afterwards.

Technique of Post-mortem Examination of Animals

152. (a) Those who are not familiar with the anatomy of various experimental animals should consult text books on the subject.

(b) Autopsy should be performed as soon after death as possible, especially if bacteriological examinations are required.

(c) Select suitable instruments and have a separate set for each step.

(d) Fasten the animal on the board exposing the ventral surface. Disinfect the hair with 1 per cent. cresol and examine the skin for ulceration, etc.

(e) Incise the skin from neck to pubis. Cut the skin at right angles at the ends of the longitudinal cuts.

(f) Separate the skin and lay the flaps back on each side, exposing the entire abdomen and thorax. Note the condition of the subcutaneous tissue, axillary and inguinal lymph glands.

(g) Make cultures of peritoneal fluid at this time if required.

(h) Examine and make cultures of abdominal organs, if necessary.

(i) With blunt pointed scissors cut through the costal cartilage making a 'V' shaped incision. Expose the thoracic organs.

(j) If blood culture is to be made lift the heart and hold it in position. Cut the pericardium and sear an area of right ventricle with a hot instrument. Make a sharp incision with a sterile scalpel, withdraw blood with a sterile pipette or a sterile loop to a suitable culture medium.

(k) If histological examinations are desired, remove small pieces of the tissue and place them in 4 per cent formol saline solution. Use 5-6 times as much solution as tissues.

(l) Dispose of the animal by burning or place it in 1 per cent. solution of crude cresol for disinfection.

SOME USEFUL PHYSIOLOGICAL AND BIOLOGICAL DATA ON LABORATORY ANIMALS

Temperature

153. For most of the experiments the temperature is generally recorded only once and in the morning at a fixed time. A clinical thermometer is suitable for monkey, sheep, rabbit, guinea pig and white rat, but for the mouse a special line thermometer has to be used. The thermometer should be pushed inside the rectum for 2" and retained for one minute. If more than one thermometer is used for recording temperatures they should be compared before hand. For lubrication liquid paraffin is ideal. Table 1 gives the normals of different laboratory animals.

TABLE 1

Animal	Limits °F	Average °F	Fever °F
Guinea pig	100–103	101·5	Over 103·0
Rabbit	100·9–104	102·4	Over 104·0
White rat	..	100·0	..
White mouse	..	99·3	..
Monkey	100–102·5	101·0	103 and over

Blood picture

154. Whenever changes in the blood picture of an animal are to be observed, before starting the experiment, a normal baseline for 4-5 days should be recorded for each animal. Table 2 gives the average normal blood cells figures for different animals.

TABLE 2

Aaimal	Red cells in millions per cmm.	White cells in thousands per cmm.	Polys %	Lympho %	Large hyaline %	Basophils %	Eosinophils %
uinea pig	5–6·5	11	42	46	6	1	5
abbit	4·5–7	04–13	45	42	9	2	2
louse	8–12	9	25	65	7·5	0·5	2
Vhite rat	8–10	8–15(12)	27	65	5	1	2
Ionkey		9–29·4 (18·490)	70	26	..	2	2

For taking blood for a count the animal should e handled gently, and the blood taken when the inial excitement is over. In monkeys the white cell ount can show an enormous rise due to excitement, ut this increase is solely contributed by polymorphonuclear cells. The lymphocytes in the same animal at different times and in different animals always remain at a constant level and hence any change in hose is significant. For these reasons the differential lood figures should be expressed in total numbers and ot in percentages, for the latter can be very misleading. Table 3 shows the degree of susceptibility of different animals to some of the important diseases.

TABLE 3

Organism	G. pig	Rabbit	Mice	Rat	Monkey	Sheep	Ferret
Pneumococci ..	+	+	+++				
Staphylococci ..		+++	++				
Streptococci ..	+	+	+	+			

TABLE 3—*contd.*

Organism	G. pig	Rabbit	Mice	Rat	Monkey	Sheep	Ferre
Meningococci ..	++	++	++		++		
Diphtheria ..	++	++					
B. tuberculosis .	++	++	++				
B. Mallei	++	++					
B. typhosum ..	++	++	++				
V. cholera ..	+	+					
B. pestis ..	++	++	++	++	++		
B. anthrax ..	++	++	++	++		++	
B. tularense ..	++	++	++				
B. Welchii	++	++					
B. oedematicus ..	++	++	++				
Typhus (ER, MR, RMSF) ..	+++	+	++	+	++		
Typhus-Indian tick ..	++			+	++		
Typhus-Scrub ..	+	++	+++	+	++		
Small-pox ..		++					
Influenza			++				+
Poliomyelitis ..			++		+++		
Dengue					+		
Mumps					++		
Measles					++		
Psittacosis ..	++	++	++		++		
Rabies		+++	+++				
Yellow fever ..	+		+		++		

+++ = Highly susceptible. ++ = Moderately susceptible. + = Suscepti

TABLE 4

Biological data of Laboratory Animals

Animal	Sexual maturity	Mating age	Breeding span	Mating ratio	Gestation period	*Litter size	Weaning	Remating after weaning
Guinea pig	0–60 days	4–6 months	0–1 year	♂–1, ♀–5	66–72 days	2, (1–6)	12–14 days or 170 G	1–2 weeks
	0–35 days		0–2 years					
Rabbit	3½–4 months	5–6 months	2 years	♂–1, ♀–1	31–32 days	8, (2–18)	6–8 weeks	3 weeks
Mice	6–8 weeks	6–8 weeks	1 year	♂–1, ♀–2†	21 days	6, (2–12)	4 weeks	4 weeks
				♂–1, ♀–5				
Rat	50–60 days	100–120 days	10–12 months		21–23 days	8, (1–17)		
			5 litters	♂–2, ♀–6†			4–5 weeks	2 weeks
				♂–3, ♀–5				
Hamster	10–15 weeks	15 weeks	9 months	♂–1, ♂–3	16–18 days	‡3–4.		
			3 litters			7, (12–14)	3–4 weeks	

Legend: —*2, (1–6) — Average 2 and limits 1 to 6. †♂1 ♀2 in case of pure lines, and ♂1 ♀5 in mixed breeds ‡8–4 in the first litter and 12 to 14 in the subsequent litters.

♂—Male ♀—Female

HORMONAL DIAGNOSIS OF EARLY PREGNANCY, HYDATIDIFORM MOLE, CHORION-EPITHELIOMA AND TERATOMA OF THE TESTICLES

155. This section is incorporated in this volume to provide the army pathologists with a ready reference regarding the methods of hormonal diagnosis of early pregnancy, hydatidiform mole, chorion-epithelioma and testicular tumours, which they are frequently called upon to perform. There are various methods described in the text books but it is hoped that if one standard method is followed the results will be much more uniform and the interpretation easier.

Principles

156. During pregnancy there is an increase in the amount of gonad stimulating factors excreted in the urine. In the presence of hydatidiform mole and chorion-epithelioma the hormone is present in urine in much larger quantities than in pregnancy. In the presence of malignant tumours of the testis the amount is also increased but is less than in pregnancy. In normal man and woman there are 5—10 mouse units of hormone per litre of urine. When a urine containing an excess of hormone is injected into female animals, eg, rabbits, mice or rats, there are visible changes in the ovaries which are not seen in case of a normal urine. The tests are stated to be reliable after the tenth day following the first missed menstrual period in suspected conception. They remain positive until 7 days after the birth of a full term baby and for 10 to 16 days after an abortion in early months. They also remain positive when the products of conception are retained. In case of testicular tumours serial quantitative tests afford an index of the efficacy of treatment.

The Aschheim-Zondek Test

157. Immature female mice weighing between 8 and 10 grams (19 to 21 days old) are employed. Every

pathologist must be able to pick up the females from the litter of immature mice. In the females there is a small dimple just below the genital protuberance and the distance between the protuberance and the anal orifice is about ½ to ¾ cm. whereas in the males there is no dimple and the distance is usually 1 cm or more. It is also necessary to use one female from the same litter as a control.

158. *The qualitative AZ Test*

(a) Collect morning specimen of urine in a clean container (sterile precautions are not necessary). In case of delay due to transit or any other reason preserve by adding 4 grs. of boric acid per 100 ml. of urine. In laboratory keep the urine on ice or in refrigerator.

(b) Filter if cloudy and adjust reaction to slightly acid if alkaline.

(c) Inject 5 animals under the skin of the back with 6 doses of 0.5 ml. of urine distributed over 2 days.

(d) 96 to 100 hours following the first injection kill the animals by anaesthetic ether and open the abdomen. It is essential to avoid bleeding. In positive cases the ovaries are enlarged, hyperaemic and show haemorrhagic and yellowish spots. The uterine horns are swollen and more transparent than normal. Histological examination of ovaries is always helpful.

If speed is indicated, eg, in ectopic gestation use eight animals. Kill four animals after 60 hours and if these are negative examine the rest after 96 hours. Positive results have been observed as early as 60 hours after the first injection.

159. *The quantitative AZ Test*—This test is usually carried out to differentiate pregnancy from hydatidiform mole and chorion epithelioma and in case of

teratoma of the testis, to determine the efficacy of the treatment. The result is expressed in terms of mouse units of hormone per litre of urine. One mouse unit is the smallest amount of urine expressed in ml that produces definite ripening of the follicles or unmistakable enlargement of the uterine horns when injections are given five times in 48 hours and animal killed in 100 hours. This amount multiplied by 1,000 gives mouse units per litre.

For example—0.2 ml. × 5 = Positive. Therefore mouse units = 1 in 1 ml. Therefore mouse units per litre = 1,000.

Immature rats can also be used for AZ Test. They should be young females 30—45 days old and not weighing over 65 gms. Only one rat is used and 0.5 ml. of urine injected subcutaneously twice daily for 3 successive days and examined after 100 hours of the first injection.

Since, in hydatidiform mole and chorion-epithelioma the amount of hormone excreted is very large it is necessary to dilute the urine in different proportions to arrive at the lowest amount necessary for a positive reaction. Similarly in cases of teratoma testis since the excretion is rather small it is necessary to concentrate the urine.

Method of dilution—The usual ranges of dilution are 1/10, 1/50 and 1/100 and sometimes 1/1000. The dilution is best done in sterile distilled water but normal saline may also be used. Diluted urine should be kept in refrigerator but should be warmed up to body temperature before injection.

Method of concentration (*Fergusson*)—(a) The fresh morning specimen is filtered if cloudy and rendered slightly acid by addition of 2 per cent acetic acid if alkaline.

(b) Add 100 ml. of 95 per cent alcohol to 20 ml. of urine in a graduated cylinder and mix thoroughly inverting the cylinder several times. The mixture allowed to stand overnight at room temperature, hormone separating out in the precipitate.

(c) Next morning, the supernatant fluid is oned off leaving about 20 ml. of fluid over the cipitate. This is centrifuged at 2,000 rpm. and the ernatant fluid is poured off.

(d) The precipitate is distributed around the bot- of the tube and allowed to dry. Add 4 ml. of dis- ed water; mix and allow to stand overnight.

(e) Next morning centrifuge this mixture. The ter now contains the hormone; it is pipetted off and ot in the refrigerator until used. This extract is imes concentrate of the fresh urine. Stronger ex- cts can be prepared by modifying this method.

A comprehensive method of estimating quantita- ely mouse units/litre from 100 to 100,000 is given page 212.

It will be evident that it is not necessary to rry out the whole range in every case. For example case of hydatidiform mole and chorion-epithelioma will be sufficient if the ranges covered by normal d diluted urine are carried out and similarly in ses of teratoma of testis the ranges covered by con- ntrated urine and normal urine should be carried t.

Therefore for any particular type of case 6 mice e used along with a control animal. The technique injection is the same as in qualitative test. Five jections are given in 48 hours the doses being ccording to the chart on page 212. After 100 hours of ne first injection animals are killed and examined nd mouse unit/litre is calculated from animal which ives a positive reaction with the minimum dose of hole urine (calculated according to amount of con- entration or dilution as the case may be).

Specimen	Dose	M.U.	Dose	M.U.	Dose	M.U.
Alcohol concentrate × 5 ..	5 × 0·4 cc	100	5 × 0·2	200	5 × 0·1	400
Normal urine	5 × 0·4 cc	500	5 × 0·2	1000	5 × 0·1	2000
Diluted urine	5 × 0·2 cc 1/10	10000	5 × 0·2 1/50	50000	5 × 0·2 1/100	100000

160. **The Friedman Test**—This test has largely replaced the qualitative AZ test with various advantages. The animal used being a rabbit the test is more clear cut microscopically. It is simpler to perform, the animal is easier to be kept ready and the result can be obtained in 48 hours. The reliability of this test is same as AZ test but it is less sensitive because the test is positive only when the urine contains at least 2,000 mouse units per litre.

The test female rabbit should not be less than 17 weeks old and should not weigh less than 1,500 gms. It should be kept segregated from other animals for 4 weeks before the test as sight and proximity of male rabbits may cause ovulation due to excitement. Some authorities prefer mature non-pregnant rabbits not less than 6 months old and weighing at least 2.5 kilos. They should have litter and isolated for four weeks preceding the test.

Methods—(a) Collect and prepare urine as under A.Z. test.

(b) Warm up the urine to body temperature and inject two doses of 10 ml each at intervals of 24 hours in the ear vein of the test female rabbit. The injection should be given slowly. A single dose of 15 ml may also be given. In case of mature non-pregnant rabbits (not recommended) two injections of 15 ml each at intervals of 24 hours should be given.

(c) After 48 hours from the first injection the animal is anaesthetized and the abdomen opened. If negative, both the ovaries remain small in size and show no change; if positive, they are enlarged hyperaemic and show 1—14 corpora haemorrhagica and corpora lutea. If one ovary is positive the other need not be examined but if one is negative the other must be examined. After examination the abdomen is closed in two layers. Some authors prefer to examine the ovaries by a dorsal midline incision or two incisions in two flanks, but these require considerable experience to perform quickly though much safer for

the animal. Others prefer to examine the ovaries before the injections but this is hardly necessary if the animal is properly isolated. It is well to remember that few rabbits are refractory to gonad stimulating hormone. In such cases the ovaries are very small and poorly developed and follicles are indistinct. In such a case, the animal should be injected with 10 ml of urine from a known pregnant case and the report withheld till the rabbit shows reaction to positive urine. If it remains negative, the test must be repeated on another animal before a final report can be given.

(d) Negative rabbits can be used immediately. Rabbits with positive results should be isolated for two weeks before use.

(e) Toxicity of the urine for animals has been occasionally found. This is mainly due to drugs. eg, Quinine, Salicylates or Santonin taken by the patient and if any history of drug intake has been obtained the urine should be detoxicated. Occasionally rabbits exhibit protein shock if the urine contains appreciable amount of albumen. In such a case the animal can be saved by intravenous injection of 0.2 ml of 1 in 1000 adrenaline.

(f) The test is known to be positive in twelve hours. In cases where speed is indicated 2 rabbits should be used. One is examined after 12 hours and, if negative, the other is examined after 48 hours.

Detoxifying method of Zondek—Filter 30 ml of urine and add 90 ml of ether, shake vigorously in a separating funnel for five minutes and allow to layer. Draw off the urine layer in an open beaker and allow to stand for sometime until the residual ether evaporates. Dissolve 0.9 gm of glucose in this urine and keep in refrigerator until used.

Good results are also obtained by washing the urine three times with equal volume of ether and then allowing all ether to evaporate from the separated urine.

Disease	Material	Animal	Method of inoculation	Period of incubation	Pathological lesions	Remarks
1. Anthra ..	Material from pustule.	Mouse or guinea pig.	Subcutaneous	1 to 2 days	Gelatinous exudate at the site of inoculation.	Bacilli particularly numerous in spleen.
2. Botulism	Filtered extract of suspected food.	Mouse or guinea pig.	Subcutaneous	1 to 4 days	Death due to cardiac or resp. failure. May show oculomotor paralysis and mydriasis.	Inoculate a control animal with antitoxin.
3. Cholera ..	Culture	Guinea pig	Intraperitoneal.	1 to 2 days	Peritonitis.	
4. Diphtheria .	Broth culture or filtrate 0·2 cc 48 hrs. old.	Guinea pig	Subcutaneous	2 to 4 days	Local oedematous swelling with enlarged regional lymph glands. Pleural and peritoneal hæmorrhagic exudate. Enlarged hæmorrhagic adrenals.	Inoculate control animal with 500 units antitoxin.

Diagnosis of Infectious Diseases by Animal Inoculations—contd.

Disease	Material	Animal	Method of inoculation	Period of incubation	Pathological lesions	Remarks
5. Plague ..	Material from bubo, sputum, or blood.	Mouse or guinea pig.	Cutaneous or Subcutaneous	2 to 5 days	Haemorrhagic lymphadenitis, caseous areas in glands, spleen and liver.	The ability to infect through unbroken skin is diagnostic.
6. Tetanus ..	Material from wound preferably filtrate from broth culture.	Mouse or guinea pig.	Subcutaneous	1 to 5 days	Tetanus. Spasticity begins in muscles nearest point of inoculation.	Inoculate a control animal and give prophylactic ATS. Rats show typical 'Seal gait'.
7. Tuberculosis	Sputum, Urine C.S.F., Exudates, etc.	Guinea pig	Subcutaneous	4 to 12 weeks	Local caseous lesions with involvement of regional and later other lymph glands. Tubercles in spleen, liver and other organs.	If material is badly contaminated treat with alkali, wash and neutralize before injection.
8. Weil's disease	Blood, urine	Mouse	Intraperitoneal	7 to 12 days	Jaundice, haemorrhages in the lungs, serous cavities, muscles, etc.	Leptospirae can be demonstrated in blood and tissues.
9. Epidemic Typhus.	Blood 3 ml	Guinea pig	Do	5 to 12 days. Fever lasting 5 to 9 days.	Tunica reaction may or may not develop. Animal recovers.	Blood, brain and spleen infective during fever. Rickettsia seen in scraping from tunica vaginalis or peritoneum.

10. Endemic Typhus.	Blood 8 cc.	Guinea pig	Do.	After 5 to 12 days, fever lasting 1-9 days.	Marked swelling of the scrotum and inflammation of the tunica.	Same.
11. Indian Tick Typhus.	Do	Do.	Do.	Do.	Do.	Do.
12. Coccidioidal granuloma	Pus	Do.	Subcutaneous	4 weeks	Typical lesions showing endogenous sporulation.	Useful in differentiation from Blastomycosis.
13. Lymphogranuloma.	Pus from bubo	Guinea pig or mouse.	Intracerebral		Encephalitis	Brain emulsion can be used for the preparation of Frei antigen.
14. Rabies	Brain or saliva.	Guinea pig or rabbit.	Subdural	Within 3 weeks.	Negri bodies in hyppocampus, fissure of Rolando, cerebellum.	Useful if suspected dog is decomposed.
15. Yellow fever	Blood (1st 3 days) liver or spleen.	Guinea pig. Mouse or Monkey.	Intracranial or intraperitoneal after intracranial injection of starch.	5 to 10 days	Encephalitis in mouse and guinea pigs. Typical lesions in viscera.	Protection tests used to detect presence of anti-bodies in epidemiological studies. Characteristic lesion in liver with intranuclear inclusions (Mastermann bodies).
16. Scrub Typhus	Blood during fever 0·5—1·0 ml.	White mice	Intraperitoneal.	5 to 14 days	Sticky peritoneal exudate, pleurisy, death.	Clusters of rickettsia can be seen in the cytoplasm of the endothelial cells from peritoneal scraping.

Diagnosis of Infectious Diseases by Animal Inoculations—concld.

Disease	Material	Animal	Method of inoculation	Period of incubation	Pathological lesions	Remarks
17. Influenza	Pharyngeal washings during the first 3 days	Ferret	Intranasal	2 to 5 days	Fever, nasal catarrh may or may not be present.	Virus is present in the lungs and nasal mucosa.
18. Poliomyelitis	Fæces or pharyngeal washings.	Monkey	Intracerebral or combined intranasal and intraperitoneal.	4 to 25 days	Fever followed by paralysis.	Virus is present in the spinal cord.
19. Small-pox	Fluid or scrapings from the vesicles.	Rabbit	Application of fluid to scarified cornea.	2 to 3 days	Keratitis characterised by crateriform lesions and diffuse corneal clouding.	Guarinieri bodies can be demonstrated in the corneal cells.
20. Virus encephalitis.	Blood or spinal fluid during early febrile stage.	Guineapig or mice.	Intracerebral	Observe for 20 days.	G.Pig—fever, weakness, tremor salivation, incordination, convulsions, paralysis. Mice—roughening of coat, increased or lack of activity, tremor, circling, hunched position, stiffening of the tail or paralysis.	

Daily Scale of Rations for Laboratory Animals/Rodents/Birds

Authority :—S.R.S. Table-Sec. IX-Table 45 as amended by amendment No. 2 of 659.

Item No.	Article	Per animal						Rodents(*a*)			Remarks
		Sheep or Goat	Rabbits	Guinea-pigs	Monkey	Dogs over 40 Lbs	Dogs under 40 Lbs	Rats per 100 or less	Mice per 100 or less	Birds per 10 or (*a*) less	
1	2	3	4	5	6	7	8	9	10	11	12
1	Ground nuts with shells. Lbs	..	0—2	0—½	0—2	..	..	0—3	0—4	..	
2	Fruits Fresh (*b*) .. .. „	..	..	..	1—0	..	..	..	..	..	
3	Vegetable fresh (*b*) .. .. „	..	0—2	0—½	..	0—4	0—3	..	..	..	
4	Potato fresh .. .. „	..	..	..	..	..	..	2—0	1—0	..	
5	Milk fresh (*c*) .. .. „	..	..	..	..	..	..	2—0	1—0	..	
6	Bread .. .. .. „	..	..	..	0—4	..	..	2—0	1—0	..	
7	Salt rock .. .. .. „	0—2	0—¼	..	..	..	..	..	..	..	
8	Eggs .. .. .. Nos	..	..	..	..	..	..	5(*d*)	3(*d*)	..	
9	Wheat .. .. .. Lbs	..	..	..	..	..	..	..	..	0—9½	
10	Bran .. .. .. „	As Required			As Required			As Required			
11	Mixed grains(*e*) .. .. „	1—0	0—2	0—2	..	..	..	..	..	0—12	
12	Dall .. .. „	..	..	..	..	..	..	..	..	0—4½	
13	Meal bone .. .. „	..	..	..	..	..	..	..	..	0—1	

Daily Scale of Rations for Laboratory Animals/Rodents/Birds—contd.

1	2		3	4	5	6	7	8	9	10	11	12
14	Salt evaporated ..	Lbs	..	..	..	..	..	..	..	..	0—$\frac{1}{2}$	
15	Lucerne green (*f*) ..	„	As required			As required			As required		0—8	
16	Onions	„	..	..	..	..	..	..	..	..	0—10	
17	Garlic	„	..	..	..	..	..	..	..	..	0—1$\frac{1}{2}$	
18	Gram whole ..	„	..	0—1	0—1	0—2	..	..	..	..	..	
19	Meat fresh (with bone) (*g*).	„	..	..	..	..	1—8	1—0	..	..	..	
20	Atta/Rice	„	..	..	..	..	0—8	0—8	..	..	..	
21	Fire wood(*h*) ..	„	..	..	..	..	1—8	1—8	..	..	..	
22	Hay bedding ..	„	As required			As required			As required			

Remarks column No. 12—

(*a*) The following instructions will apply in respect of scales of rations for Rodents and Birds:—

(I) The scale for each additional rat/mouse in excess of 100 is 1/100th of the scale laid down in columns 9 and 10. The scale for each additional bird in excess of 10 is 1/10th of the scale laid down in column 11.

(II) When the total daily entitlement works out to a fraction of an ounce/number it will be rounded to the nearest ounce/number.

(III) The unit may resort to local purchase of such items as are certified by ASC as unavailable and claim the cost thereof on a contingent bill in respect of rations for birds.

(*b*) Variety of fruit fresh and vegetable fresh required will be stated in demand and any variety of ASC supply so demanded will be supplied.

(*c*) When milk fresh is not available, tinned milk will be supplied in lieu.

(*d*) When the total daily entitlement works out to a fraction it will be rounded to the nearest whole.

(*e*) Composed of equal parts of wheat, barley, gram and bajra.

(*f*) 50 per cent of grass may be taken in lieu.

(*g*) Milk may be issued to sick dogs against an equivalent quantity of meat fresh on the recommendation of a Vet. Officer.

(*h*) For cooking chappatties, rice, meat and vegetables for dogs.

PART IV

MICROSCOPE

L/B(D)100--18

MICROSCOPE

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MAINTENANCE OF MICROSCOPE

Introduction

161. A microscope which is like a pair of powerful eyes to the scientist is a sturdy instrument with delicate optical parts. It stands prolonged use if care is exercised. Day to day attention in the course of work is the best way for proper maintenance of such an instrument. It is an instrument of precision and care must be taken to preserve its accuracy. Besides acquiring the ability to use an instrument properly with its accessories, it is important to know how to keep it in the best working condition.

Day to day care

162. Each microscope when issued by the manufacturers is accompanied by a handbook of instructions. These instructions should be scrupulously followed by the user. Certain general rules which apply to the use of all microscopes are—

(a) the microscope case should be dusted before the microscope is taken out.

(b) the microscope should be handled by the upright body and not by any other part.

(c) the microscope should not be handled with force or in any way subjected to jarring.

(d) the exterior surface of the eye piece, the objective, the condenser and the reflecting mirror are apt to collect dust. Hence, when not in use, the microscope should be kept either in its case or covered with a bell jar or kept under a soft clean cloth loosely thrown over it. If dust collects on any glass surface it should be removed with a soft brush and

then the gass surface wiped with old cotton cloth. Dust contains minute particles and if rubbed on the surface while cleaning, the glass surface may become scratched. Optical glass is softer than window glass and can be easily damaged.

(e) glass surface should not be handled with fingers; grease marks are easily left by fingers on glass surface.

(f) the lenses should not be cleaned by blowing on them. Cedar wood oil should be removed from the oil immersion lens at the end of the day; there is no advantage in doing it oftener. The oil can be removed with xylol which should be applied to the lens surface with a piece of clean silk or with clean soft linen. The cleaning should be done by a rotating movement.

(g) alcohol is never to be used to clean the metal parts of a microscope for the lacquer paint is partially soluble in alcohol. The dry cloth or a cloth moistened with water should be used for the painted parts; xylol may also be used to clean lacquered parts.

(h) the cedar wood oil should be perfectly clear for use. The depth of the oil in the bottle should be so adjusted that the rod when lifted out will carry the right sized drop of oil and no excess will fall on the table or on the microscope. If the cedar wood oil becomes very thick, it should be discarded.

(i) the object should first be spotted by the help of the low power objective and then the high power is used. The immersion lens is brought into use as required.

(j) the condenser is generally centered at the time of the manufacture, but if it goes out of alignment, the centering screws may be used to adjust it.

(k) no part of the microscope may be used with force; if any part becomes stiff, it should be oiled very thinly with paraffin or microscope lubrication oil.

(l) the instrument must be kept clean at all stages.

163. **Detailed attention to parts**

(a) *Care of the stand*

(i) Dust should be removed by soft brush and then by a wet cloth. The wiping should be done with the grain of the finish of the metal and not across it.

(ii) To use the draw tube, where an adjustable draw tube is part of an instrument, use spiral motion in adjustment.

(iii) If a screw driver has to be used on an instrument its two surfaces should be ground parallel and not left wedge-shaped. It is, however, better to leave such use to an instrument repairer.

(b) *Joint for inclination*

If the joint should become loose so as to prevent the arm being set at any angle of inclination, it should be tightened by drawing up the nut at one or the other side. If the nut has a screw slot, a properly prepared screw driver should be used but if it has two holes a suitable key should be obtained from the maker. In high grade instruments the axle is generally tapering and to determine which nut is to be drawn up can only be done by trial.

(c) *Care of the coarse adjustment*

Special care should be taken to keep the coarse adjustment free from dust. The slides and the rack and pinion are exposed while working and the lubricant is apt to catch dust and gum. The tube should be occasionally withdrawn from the arm and the slides carefully wiped with a cloth moistened with Xylol. Lubrication should be done by applying a small quantity of paraffin oil to a cloth and wiping well over the surfaces. Excess of oil should be removed with a dry cloth. The teeth of rack and pinion should not be lubricated. An occasional cleaning of the teeth with a tooth brush is advisable.

It is also advisable from time to time to lubricate the pinion shank on both sides of the arm with minute quantity of paraffin oil.

If the pinion works loose owing to jarring during transportation or by long use, and the body does not remain in position, the friction can be increased by tightening the two screws on rack slide.

(d) *Care of fine adjustment*

If the fine adjustment does not respond to the turning of the micrometer screw, or if it comes to a stop, it indicates that the adjustment screw has come to the limit of its motion at either end. It should by no means be forced; it should at all times be kept at a medium point. If the fine adjustment ceases to work satisfactorily, the instrument should be sent for repairs.

(e) *Care of objectives and eye pieces*

Dust should never be permitted to settle on the lenses nor should the finger be allowed to come into contact with any of the surfaces. At regular intervals the eye piece should be

unscrewed and the internal surface cleaned with a soft hair brush or camel's hair brush or a well washed piece of linen or silk. Chamois leather can also be used for cleaning after repeated washings.

It is not desirable to open the objective lens.

(f) *Mechanical stage*

The screws fixing the microscope should not be disturbed. If cleaning of the stage becomes necessary, then the screws should be taken off gently and replaced after cleaning, taking care that no thread is forced.

(g) *The condenser and the reflecting mirror*

These are apt to collect dust very easily and they should be cleaned by a soft cloth.

(h) *Filters*

All microscopes are issued with filters and should be cleaned by wiping with a soft cloth. When not in use, they should be kept within the microscope box in their appropriate cover.

(i) *Iris diaphragm*

This is generally carried in the frame of the condenser. Its manipulation should be done gently, otherwise the thin leaves may become entangled with one another. If any distortion occurs in the diaphragm it should immediately be sent for repairs.

(j) *The microscope lamp*

Though normally it is not a part of the microscope, it often is an accessory. The light, if fixed properly once, need only be switched off and on, and dusted. For dark ground work where a carbon arc lamp is used, the microscope and the lamp should not be disturbed from their relative positions, once adjustment is made.

164. Some Don'ts in Microscopy

(a) Don't rack the body down on the object.

(b) Don't use the rack when the object glass is very close to the object; use the fine adjustment.

(c) Don't force any of the adjustments.

(d) Don't touch any of the lens surfaces with the fingers, they always leave a grease mark.

(e) Don't leave immersion oil on a lens surface, wipe it off with benzol or xylol.

(f) Don't leave the microscope in a damp or dusty place.

(g) Don't attempt to take object glasses to pieces. Eye piece lenses may be unscrewed and cleaned with silk.

(h) Don't let immersion oil get on the stage or any adjustment; it gums them up.

(i) Don't use any lubricants except those specially made for the instrument.

(j) Don't use too strong a light.

(k) Don't use a high power if a low power will show what is desired.

165. Some Do's in Microscopy

(a) Do wipe the instrument clean before use on every occasion.

(b) Do keep the microscope under a bell jar or a cloth cover while on the table but not in use.

(c) Do clean the oil immersion lens after the day's work.

(d) Do work with both eyes open, both physically and otherwise.

(e) Do consider the microscope as the most valuable aid of the pathologist.

PART V

PARASITOLOGY

PARASITOLOGY

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INTRODUCTION

166. This section deals with the various methods employed in the diagnosis of protozoal and helminthic infections. Depending on the nature of the infection, various materials will have to be examined in order to arrive at a diagnosis. Thus, examination of blood will be necessary for the specific diagnosis of Malaria, Trypanosomiasis, Leishmaniasis or Filariasis. In the case of intestinal protozoal or helminthic infections, the faeces are examined. Urine examination may reveal the ova of schistosoma or microfilariae. Examination of the sputum may be of value in the diagnosis of amoebic abscess of the lung or hydatid cyst. Biopsy material removed from various sites may be necessary for the diagnosis of several parasitic infections. The diagnosis of some parasitic infections is facilitated by biochemical and immunological tests which are dealt with under the appropriate sections.

CLASSIFICATION OF PROTOZOA

167. For purposes of diagnosis by laboratory methods, human parasitic diseases will be dealt with under two main heads—Protozoa and Helminths. The phylum protozoa, which are of medical importance, may be classified as under—

1 Sub-phylum	Plasmodroma
1. Class	Rhizopoda
Genus and species	Entamoeba histolytica
	Entamoeba coli
	Endolimax nana
	Iodamoeba butschlii
	Dientamoeba fragilis

2. Class	Mastigophora
A. *Haemoflagellates*	
Genus and species	Trypanosoma gambiense
	Trypanosoma rhodesiense
	Trypanosoma cruzi
	Leishmania donovani
	Leishmania tropica
	Leishmania braziliensis
B. *Intestinal flagellates*	
Genus and species	Trichomonas hominis
	(T. vaginalis in vagina)
	Enteromonas hominis
	Embadomonas intestinalis
	Chilomastix mesnili
	Giardia intestinalis
3. Class	Sporozoa
Genus and species	Plasmodium vivax
	Plasmodium falciparum
	Plasmodium malariae
	Plasmodium ovale
II Sub-phylum	Ciliophora
Class	Ciliata
Genus and species	Balantidium coli

CLASSIFICATION OF HELMINTHS

168. The phylum Helminths, which are of medical importance, may be classified as under—

I Sub-phylum	Nemathelminthes
Class	Nematoda
Species	Ascaris lumbricoides
	Ankylostoma duodenale
	Strongyloides stercoralis
	Enterobius vermicularis
	Trichuris trichiura
	Trichinella spiralis
	Wuchereria bancrofti
	Wuchereria malayi
	Dracunculus medinensis
II Sub-phylum	Platyhelminthes
1. Class	Cestoidea
Species	Taenia saginata
	Taenia solium
	Hymenolepis nana
	Diphyllobothrium latum
	Echinococcus granulosus

2. Class	Trematoda
Species	Fasciolopsis buski
	Paragonimus Westermanni
	Schistosoma haematobium
	Schistosoma mansoni
	Schistosoma japonicum

DIAGNOSIS OF PROTOZOAL INFECTIONS

169. According to the nature of the infection different material will have to be examined for the particular parasite. Blood and faeces constitute the main sources of the parasite but in certain cases it may be necessary to examine biopsy material from a lymph node, the spleen or bone marrow.

170. **Rhizopoda**—The protozoa belonging to this group are found in the intestines, and examination of the faeces is necessary in order to establish their presence. The trophozoite or cyst of the particular parasite may be seen. Entamoeba histolytica is the only pathogenic member of this group; the presence of other rhizopoda in faeces is merely incidental.

171. *Collection of faeces*—In a case of acute amoebic dysentery the diagnosis depends upon the demonstration of vegetative forms of Entamoeba histolytica in the faeces. These organisms do not survive long outside the body and soon lose their motility and morphological characters, when cooled. The stool of the patient collected in a clean bed pan free of disinfectant should be sent to the laboratory as soon as possible for examination. If the patient is passing formed stools, cysts will probably be found but not the trophozoites.

172. *Macroscopic examination*—The nature of the stools in acute amoebic dysentery will vary according to the extent of the intestinal lesions. Ordinarily there is blood and mucus intimately mixed with faecal matter. Compared to the stool in Bacillary dysentery it is relatively copious in bulk with an offensive odour. It is acid in reaction.

173. *Microscopic examination*—A small flake of mucus is picked up with a wire loop or swab stick and emulsified on a clean glass slide with a drop of previously warmed normal saline. A clean cover glass that has been ringed with vaseline is placed over the emulsified stool and pressed down lightly so as to make an airtight seal. This can be placed in an incubator for a few minutes to stimulate the amoebae into movement, without fear of the stool becoming dry on the slide.

In acute amoebic dysentery, many motile amoebae containing ingested red blood corpuscles will be seen. The cellular exudate consists of red cells in clumps with a few pus cells, macrophages and epithelial cells. Charcot Leyden crystals may be seen but they are not pathognomonic of amoebic dysentery.

Entamoeba histolytica is a unicellular organism which is on an average 30 microns in diameter. Its shape constantly changes due to the pseudopodia which it throws out to effect its motility. The cytoplasm can be divided into two portions, a clear ectoplasm and somewhat dense endoplasm. The nucleus, which is contained within the endoplasm, is about 5 microns in diameter and is bounded by a membrane on the inner side of which are arranged regular dots of chromatin; inside the nucleus is a prominent central karyosome. Within the endoplasm are found leucocytes, tissue debris and several red cells. This erythrophagocytosis at once differentiates Entamoeba histolytica from Entamoeba coli and other non-pathogenic rhizopoda.

Entamoeba coli is about the same size as E. histolytica but the cytoplasm is not similarly differentiated. The chromatin granules on the nuclear membrane are coarser and the karyosome eccentric. Cytoplasmic inclusions include food vacuoles, yeast and bacteria, but never red cells. Its motility is sluggish compared to that of E. histolytica.

Other rhizopoda viz Endolimax nana, Iodamoeba
utschlii and Dientamoeba fragilis, are seldom greater
nan 10 microns in diameter and never contain red
ells in the cytoplasm.

PROTOZOAL CYSTS

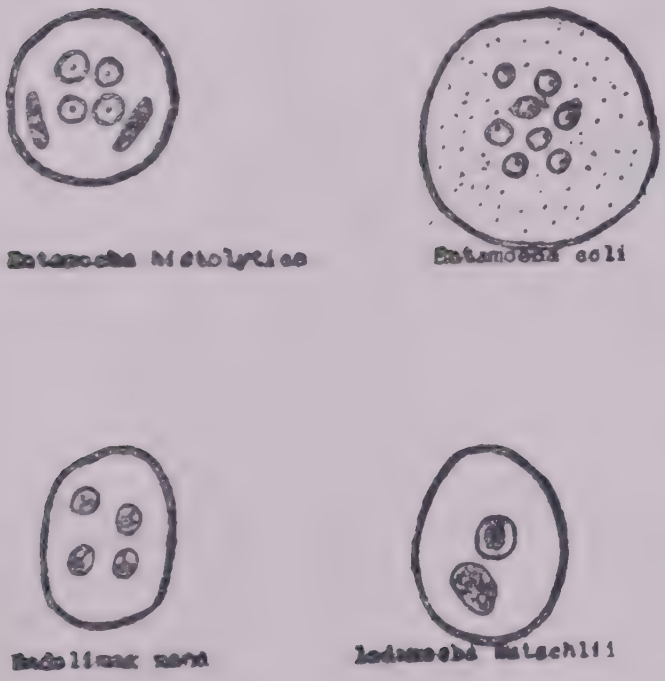

FIG. 1

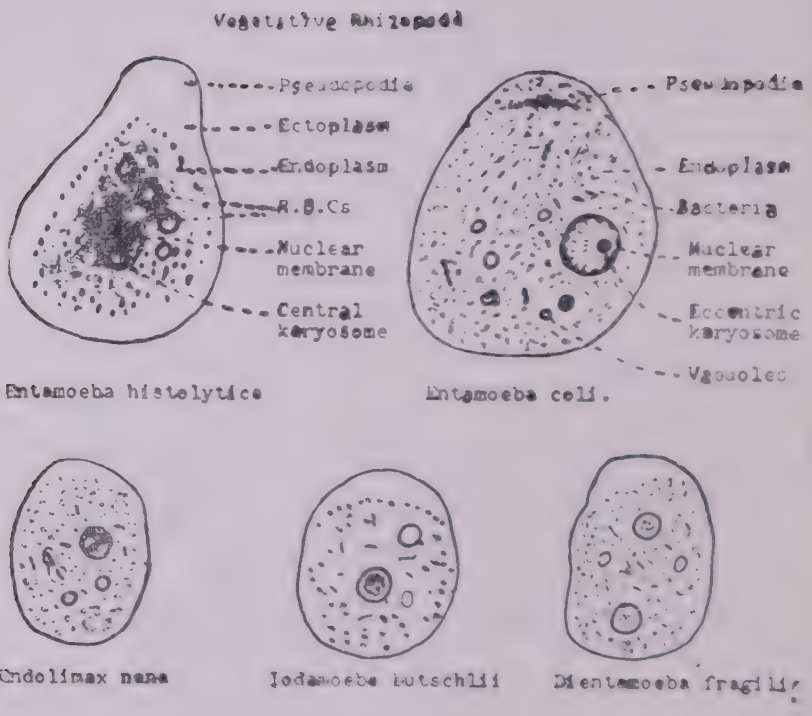

FIG. 2

174. *Preparation of permanent smears*—In order to make permanent stained preparations of Entamoeba histolytica, a small flake of mucus, from a fresh stool of amoebic dysentery known to contain amoeba, is spread thinly on a clean glass slide. The still moist slide is placed immediately in a jar containing Schaudinn's Solution and allowed to become fixed in this solution for 5—10 minutes. The smear is then stained with Heidenhain's haematoxylin according to methods described under the section on histopathology.

175. *Stained smears for protozoal Cysts*—A few drops of Lugol's iodine solution is placed on a clean glass slide and a loopful of faeces is emulsified in it. Emulsification should be sufficient to provide an

evenly spread smear. This is covered with a vaseline ringed coverglass and examined. Iodine is lethal to vegetative protozoa and is not recommended for their identification, but it is extremely valuable in the identification of cysts as the nuclei and glycogen masses become stained with iodine and allows nuclear identification which remain almost invisible in saline preparations. The chromatoid bars of E. histolytica or E. coli are not stained by iodine.

A small portion of stool placed in three or four times its volume of iodine solution can be stored as such and examined at some later convenient time, when it will be found that the nuclei and glycogen have been stained.

176. *Concentration of cysts*—Thoroughly emulsify about a gram of faeces in about 5 ml of distilled water in a small tube. Centrifuge at about 2000. r.p.m. for one minute. Decant the supernatant fluid and add fresh water to the same level in the tube, emulsify thoroughly and centrifuge as before. Repeat the process until the supernatant remains clear. Decant the supernatant and add 33 per cent Zinc Sulphate to fill the tube to within $\frac{1}{4}$ inch from the top and mix thoroughly. Centrifuge at 2500 r.p.m. for about a minute and with a wire loop remove a drop of fluid from the surface and place on a clean glass slide; apply a coverglass and examine. An iodine preparation should also be made at the same time and examined.

177. *Identification of cysts*

1 *Entamoeba*—

(i) *E. histolytica*—The mature cyst varies in size from 9 to 20 microns in diameter. The cyst wall is clear and the cytoplasm hyaline. There are normally four nuclei each 2 microns in diameter and having the same characters as the nucleus of the trophozoite. In the cytoplasm there are usually two oblong bars with rounded

ends called chromatoid bars and a glycogen mass which stains brown with iodine.

(ii) *E. coli*—The cyst is round and measures 15 to 20 microns in diameter. The wall is slightly thicker than E. histolytica and the cytoplasm is granular. There are usually eight nuclei about 2 microns in diameter each resembling the nucleus of the trophozoites. A small glycogen mass may be present. Chromatoid bars are usually absent but if present they have ends resembling splintered glass.

2 *Endolimax*—
The cyst of Endolimax nana is oval in shape and 8 to 9 microns in diameter. The cytoplasm is granular and has four nuclei each having an irregular karyosome connected by a filament to another small mass. There are no chromatoid bars or glycogen mass.

3 *Iodamoeba*—
The cyst of Iodamoeba butschlii is oval in shape and 8 to 12 microns in diameter. It has large dense glycogen mass. There is only one nucleus eccentric in position containing a karyosome which is surrounded by refractile globules. There are no chromatoid bars.

4 *Dientamoeba*—
The cysts of Dientamoeba fragilis have not been demonstrated.

Flagellated Protozoa

178. In the parasites belonging to this group movement is carried out by means of one or more flagella. Those which infect the blood and various tissues of the body are called haemoflagellates and include trypanosoma and leishmania. Human trypanosomiasis, however, has not been reported in India. Those which infect the intestines are called intestinal flagellates.

Haemoflagellates

179. *Trypanosomes*—The diagnosis of trypanosomal infection is based upon the demonstration of the parasite in the localised skin lesion, the blood, aspirated fluid from a lymph node, spleen, bone marrow biopsy, cerebrospinal fluid, or by cultivation of the organism or even by animal inoculation.

Blood films—Trypanosomes can be demonstrated in thin blood films stained with J.S.B., Leishman or Giemsa stains. Where the parasites are scanty it may be necessary to prepare a thick blood film for examination.

Concentration method—0.5 ml of blood is collected in 4.5 ml of 3.8 per cent Sodium citrate in a centrifuge tube and centrifuged at 1000. r.p.m. for 10 minutes. Withdraw the leucocytic layer and supernatant into another tube and centrifuge again at 2000. r.p.m. for 15 minutes. Pour off the supernatant fluid, remove a small amount of the deposit and make a smear and stain as for a blood film and examine.

Cerebrospinal fluid—2—5 ml of cerebrospinal fluid is centrifuged for 10—15 minutes at 2000. r.p.m. and the stained deposit examined for trypanosomes.

Animal inoculation—Monkeys, rats, mice or guinea pigs may be inoculated with infective material in order to demonstrate trypanosoma. For this purpose blood is mainly used for inoculation into these animals but aspirated fluid from a lymph node or cerebrospinal fluid may be used. When blood is used to inoculate laboratory animals, 10 ml of blood is withdrawn from the patient and put into a tube containing 2 ml of 3.8 per cent sodium citrate, which is immediately shaken to prevent clotting.

2.5 ml is given intraperitoneally into each of two guinea pigs, 1 ml is given intraperitoneally into each of four rats and 0.5 ml is given intraperitoneally into each of four mice. The blood of these animals is

examined daily for a month or more because the trypanosoma may have no effect at all on the animals, they may produce a chronic infection or cause a very rapidly fatal disease. By examining the blood of all the animals daily it is most unlikely that the trypanosomes will be missed whatever course the disease should take.

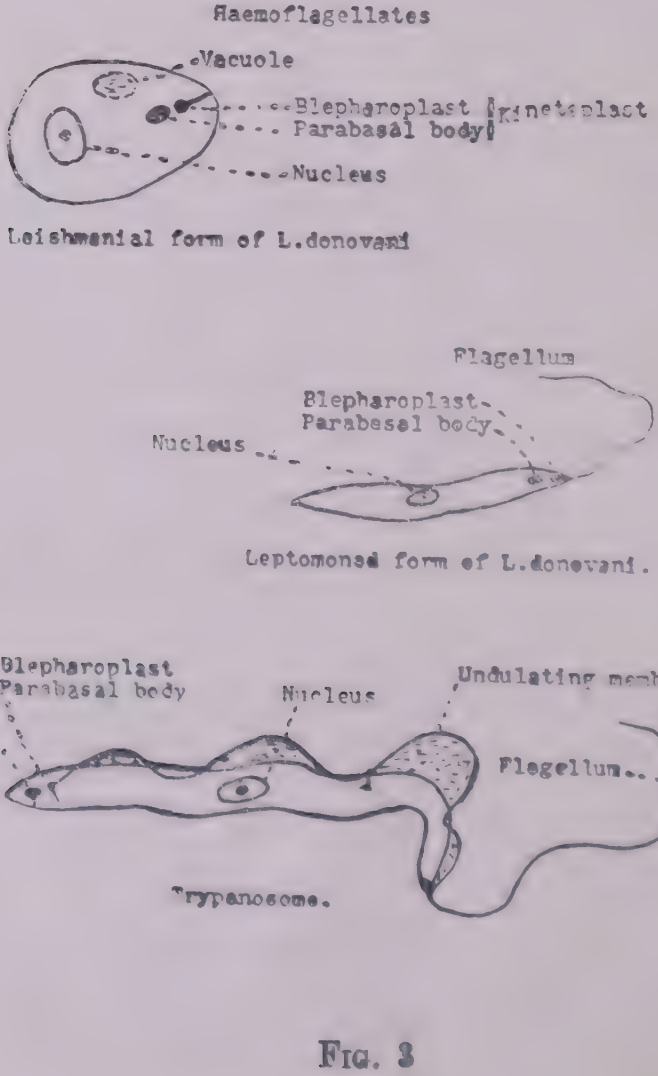

FIG. 3

180. *Leishmania*—There are three species of Leishmania parasitic to man, namely Leishmania donovani which causes Visceral Leishmaniasis, Leishmania tropica which causes cutaneous Leishmaniasis and Leishmania braziliensis which causes Espundia, a localised lesion of the skin and nasopharyngeal mucous membrane. All three species are morphologically identical and exist in the leishmanial form in man and in the leptomonad form in culture or in the insect host. The leishmanial or aflagellar forms are oval bodies about 2—4 microns in their long axis. The nucleus is oval and situated at the side of the cell wall. Almost perpendicular to the nucleus is the kinetoplast which is made up of a rod-shaped parabasal body and a rod-like blepharoplast. At one side of the cell is a vacuole. The leptomonad form obtained in culture and also in the insect host is a spindle shaped body about 15—20 microns long and 1-2 microns in breadth. There is a single flagellum at the anterior end. The nucleus is central and the kinetoplast at the anterior end.

181. *Leishmania donovani*—In cases of Visceral Leishmaniasis this organism can be demonstrated in the bone marrow, spleen and lymph nodes and sometimes in liver puncture and peripheral blood. Culture of this material can also produce the flagellate forms of this parasite.

Sternal Puncture—Material obtained by sternal puncture offers a sure method for the diagnosis of Visceral Leishmaniasis particularly where the spleen is not sufficiently enlarged to be punctured. It is considered a safer procedure than splenic puncture. If the parasites are scanty, examination of sternal bone marrow may give negative results. Using all sterile precautions the marrow cavity of the Sternum at the level of the 2nd or 3rd intercostal space is punctured by means of a sternal puncture needle and 1/2 to 1 ml of marrow is withdrawn into a dry sterile 2 ml

syringe. The material thus withdrawn is put into a sterile waxed watch glass. 1-2 mm pieces of marrow are picked up with sterile forceps and placed on a microscope slide. Using another clean slide the marrow is pressed between the slides and drawn out to make a smear. By this means several smears can be prepared. Smears that are not stained immediately should be fixed in methyl alcohol for 3—5 minutes.

Some marrow particles can also be inoculated into N N N medium for cultural examination.

Splenic puncture—The parasite can be found in stained films of material obtained from splenic puncture.

Liver Puncture—This method may be employed being safer than splenic puncture, but the parasites are more difficult to find. This method therefore offers no advantages over other methods in the diagnosis of visceral Leishmaniasis.

Blood—Examination of a stained blood film is sometimes successful in demonstrating the parasite in the peripheral blood, usually phagocytosed within a neutrophil leucocyte or a monocyte. These cells are found in large numbers in the tail end of a blood film. While making a thin blood film, if the spreader is lifted off abruptly before the blood on the slide is completely exhausted, a straight edge containing a large number of leucocytes is produced. In a suspected case of Visceral Leishmaniasis the straight leucocytic edge is examined for presence of the parasite.

Culture—The parasite can be cultured from the blood and from material obtained by sternal or splenic puncture. In the case of peripheral blood, 2 ml is diluted with 10 ml of sterile citrated saline (2 per cent sodium citrate in normal saline). After having been allowed to settle overnight at 22°C, the cellular deposit is removed aseptically with a sterile Wrights pipette and inoculated into the water of condensation

of 4—6 tubes of N N N medium. A drop of condensation fluid is examined after about 10 days and thereafter at weekly intervals. In positive cases actively motile leptomonad forms are seen. Bacterial contamination inhibits the growth of this parasite, therefore, strict asepsis must be observed throughout the procedure.

Material obtained by sternal, lymph node or splenic puncture can be inoculated direct in small quantities into N N N medium.

182. *Leishmania tropica*—This is the parasite which causes cutaneous leishmaniasis or Oriental sore. It is found within the reticulo-endothelial cells of the skin and not in the bone marrow, liver, spleen or lymph node. It can be demonstrated by making a smear of the material obtained by aspiration of the indurated edge of the sore and staining it with JSE Leishman or Giemsa stain.

Preparation of smear

The indurated edge of the sore is cleaned with spirit and allowed to dry. It is then pricked with a cutting needle and the serosanguinous fluid which oozes out is collected with a Wright pipette and a drop is placed on a clean microscope slide and a smear prepared and stained.

Culture—The material obtained for the preparation of a smear can be inoculated direct into N N N medium. Strict asepsis must be observed throughout the procedure.

183. *Leishmania braziliensis*—This parasite is the cause of mucocutaneous leishmaniasis or localised ulceration of the skin and the mucous membrane of the nose, mouth and pharynx. Infection with this parasite has not been reported in India. The organism can be demonstrated in the macrophages of the skin and mucous membrane of these sites. Smears should be made from aspirated material from the lesions and

stained with JSB, Leishman or Geimsa's stain. If obtained under sterile precautions this material can also be inoculated into N N N medium for isolation of the parasite.

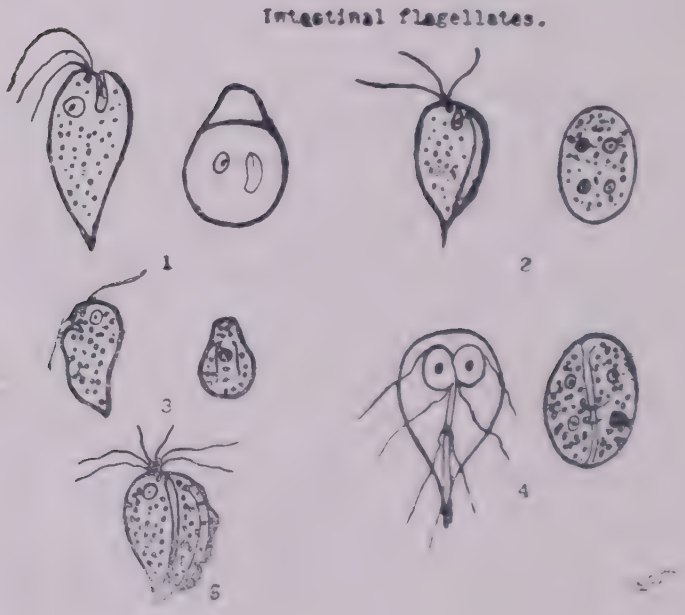

FIG. 4—Intestinal flagellates

Trophozoites and cysts of 1. Chilomastix mesnili. 2. Entermonas hominis. 3. Embadomonas intestinalis. 4. Giardia intestinalis. 5. Trichomonas hominis (no cystic forms).

Intestinal Flagellates

184. These flagellated protozoa inhabit the intestines of man. There are five species each belonging

to a separate genus, viz Chilomastix, Trichomonas, Enteromonas, Embadomonas and Giardia. All of these flagellates, with the exception of Trichomonas, exist as trophozoites and cysts. They are adapted to live in a fluid or semi-fluid environment, and are therefore usually seen in diarrhoeic stools.

Microscopic examination—For the diagnosis of intestinal flagellate infection, examination of a fresh specimen of stool is required. A small portion of faeces is picked up on a wire loop and emulsified in a drop of normal saline on a slide. This is covered with a cover glass and examined. For identifying cysts, the stool is emulsified in Lugol's iodine, the cysts can also be concentrated as for E. histolytica cysts.

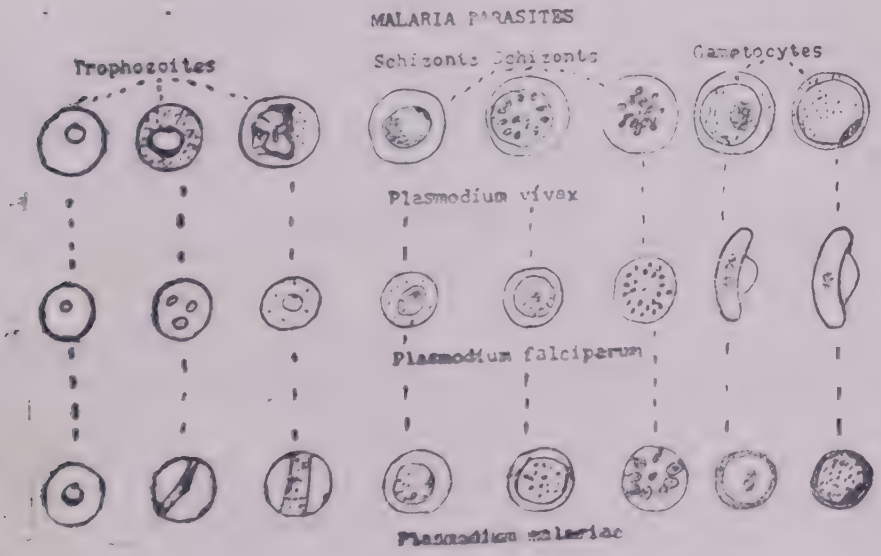

FIG. 5

Sporozoa

185. Under this class are included the malarial parasites of which there are a large number of species. Some are parasitic to apes, monkeys, birds and cold blooded animals while others are parasitic to man. There are three species of malarial parasites which are parasitic to man in India viz Plasmodium malariae, Plasmodium vivax, and Plasmodium falciparum. Plasmodium ovale is essentially an African species and not found in India. As the sexual cycle of these parasites occurs in the red blood cells, a diagnosis of malaria can easily be arrived at by an examination of the peripheral blood. A diagnosis of clinical malaria should always be confirmed by demonstration of the parasites in a blood film.

186. *Examination of the peripheral blood*

Thin film—A film is made on a microscope slide in exactly the same way as for a differential count and stained with JSB, Leishman or Giemsa. The technical details attached to the preparation of a good blood film and the methods of staining are very important and have been dealt with under the section on Haematology.

When the film has been stained, a preliminary examination of it under low power of the microscope will indicate whether the smear has been stained properly or not. If the leucocytes in the film appear to have been stained correctly then it may be safely assumed that any malarial parasites present in the film will also have been stained satisfactorily. When searching a thin film for parasites the upper and lower margins of the tail of the film should be particularly examined as the parasites tend to be more numerous at these sites in the film. At least one hundred fields should be examined before declaring a smear negative for malarial parasites.

Thick film—In most cases of malaria, a single thin film will reveal the presence of the parasite, but during the first two days of a primary infection and in P. falciparum infection, particularly in cases of cerebral malaria, the parasites may be very scanty and often missed in a thin film. Under these circumstances it will be necessary to prepare a thick film, which is nothing more than a concentration method for demonstrating parasites in the blood. The parasites can easily be seen in a thick film but for identification of the species it may be necessary to see a stained thin film.

A thick film is prepared by taking a large drop of blood in the centre of a clean glass slide and spreading it out to form an area of a half inch square with the corner of another slide or with a needle. The film is kept covered with a petri dish during the process of drying.

Staining of thick films—The film may first be dehaemoglobinised by standing vertically in a koplin jar of distilled water for 5 to 10 minutes. It is then allowed to dry and stained by JSB, Leishman's or Giemsa's stain in the ordinary way as for thin films.

Alternatively the blood cells can be laked and stained simultaneously by any of the three stains mentioned above.

For satisfactory laking of the blood in a thick film, it should preferably be a freshly made smear. During hot weather and when the films are allowed

to stand overnight they tend to get fixed and laking is seldom as complete as in a fresh film. A thick film fixed with alcohol is useless for laking purposes and should be avoided.

J.S.B. Stain—The film is placed in solution 1 for 10 seconds and then in acidulated tap water for 2 seconds. It is then stained in solution II for 2 seconds and then returned to solution I again for 10 seconds. The film is then washed for 10 seconds and allowed to dry and examined.

A 1 in 10 dilution of Leishman's stain in distilled water or a 1 in 10 dilution of stock Giemsa's stain in M/15 Phosphate buffer of a pH of 7.2 is poured on to the thick film and left for 30 to 40 minutes and then carefully washed in tap water and allowed to dry.

When thin and thick blood films are made on the same slide, care should be taken when staining the thin film that no undiluted Leishman's stain is allowed to come in contact with the thick film as this will fix it and render it valueless for subsequent examination. When the Leishman's stain on the thin film is diluted with two volumes of distilled water, the diluted stain may then be flooded over the thick film. When staining with Geimsa's stain the thin film is first fixed by immersion in methyl alcohol for 3 to 5 minutes and then both films are flooded with 1 in 10 Giemsa in Phosphate buffer as described above.

Differential features of the Malarial Parasites

	P. falciparum	P. vivax	P. malariae	P. ovale
Schizogonous cycle Trophozoite	36–48 hours 1·5 microns. Fine blue cytoplasmic ring. Some have two chromatin dots. Accole forms and multiple infection common	48 hours 3 microns. Thick ring. Growing forms irregular	72 hours 2 microns. Thick ring. Vacuole not prominent. While growing assumes band or comet forms	48 hours. 3 microns. Thick ring with inconspicuous vacuole.
Mature Schizont	Not seen in blood—has 24 merozoites.	10 microns. Has 12–24 merozoites	7 microns. Has 6–12 merozoites	6 microns. Has 6–12 merozoites.
Gametocyte	Crescentic Ghost cell faintly seen	Globular Schuffners dots present	Round	Schuffners dots present.
Infected RBC	Unaltered Maurer's dots seen	Enlarged	No change No pigment	Unaltered. Schuffners dots seen.

L/B(D)100—20

Ciliata

187. As the name implies this class of parasite moves by means of cilia. There is only one species although rarely of importance to man viz, Balantidium coli which causes ciliate dysentery or balantidiasis. The parasite is quite commonly found in the intestines of pigs in which it leads a harmless existence. In man, however, it gives rise to ulceration of the colon almost indistinguishable from amoebic ulcers.

The diagnosis of this condition is established by microscopical examination of the faeces. In the acute stage of the disease the trophozoites and in chronic cases the cysts are found.

In preparing a smear of the stools for examination the same technique is employed as in the case of examination for other intestinal protozoa.

The parasite is identified by its characteristic morphology. It is oval in shape and 70 microns in length. The whole body is covered with cilia. At one end of the organism is a rudimentary mouth. It has a large crescentic macronucleus and a small round micronucleus. Several contractile vacuoles may be seen. The cyst is 50 microns in diameter with two nuclei as in the trophozoite form with one or two contractile vacuoles.

Balantidum coli

TROPHOZOITE CYST

FIG. 6

DIAGNOSIS OF HELMINTHIC INFECTIONS

188. The diagnosis of helminthic infections depends upon the identification of the helminth. If due to its particular localisation in the body it is not possible to gain access to it, one has to depend upon examination of the various excretions of the body or even the blood whereby the ova or larvae of the worms may try to gain exit from the body in order to perpetuate its existence in a new host. It may be necessary, therefore, to examine the blood, faeces, urine, sputum or biopsy material from various parts of the body in order to establish a firm diagnosis.

Nematodes

189. These worms are elongated, unsegmented and filiform in appearance which vary in size from a quarter of an inch to about 3 ft in length. The sexes are separate, the male always being smaller. Some nematodes are viviparous such as Wucheraria bancrofti and Trichinella spiralis, others are oviparous. All pass their life history in one host except Dracunculus medinensis which requires a fresh water crustacean Cyclops for development of its larvae, and Wucheraria whose larvae are taken up from the blood by a mosquito. The Cyclops and mosquito are referred to as intermediate hosts.

Intestinal Nematodes

Intestinal nematode infection is diagnosed by finding the worm, its ova or larvae in the faeces.

190. *Ascaris Lumbricoides*—This worm somewhat resembles the ordinary earthworm. They vary in size from 15 to 40 cms, the male being smaller than the female with a hooked posterior end. The ova are usually oval in shape and 60 to 70 microns in its long diameter. They are brown in colour due to bile staining and have a mamilated outer shell. The eggs float in saturated saline. Diagnosis is simple by demonstration of eggs except when infection is limited to male worms.

191. *Ankylostoma duodenale*—The adult worms are cylindrical in shape and creamy white in colour.

the anterior end being bent dorsally. Their length varies from 8 to 12 mm by about half a mm in breadth. The posterior end of the male is dilated into what is termed a copulatory bursa. The eggs are oval in shape and colourless or slightly grey. They measure about 60 microns in their long diameter; inside, the ovum is seen to be composed of four segments which are separated by a clear space from the shell.

192. *Strongyloides stercoralis*—This nematode is just visible to the naked eye being about 2 mm long by 50 microns wide. They live in the intestines but may also be found in the lungs (Somatic nematode). The eggs are about the same size as those of Ankylostoma and contain a larva ready to hatch. The larva is liberated almost immediately after the egg is laid and it is the presence of the larvae in the faeces or sputum which is diagnostic of infection with this parasite. The larvae in the faeces are about 200 microns in length by 15 microns in breadth.

193. *Enterobius vermicularis*—The length of this nematode varies from 4 mm to 12 mm. In the male the posterior end is sharply curved and in the female it is long and tapering. The ova are asymmetrically oval in shape and measure about 50 microns in its long diameter. The gravid female lives in the rectum but deposits its eggs on the skin just outside the anus. The ova are therefore not usually found in the faeces.

194. *Trichuris trichiura*—Morphologically these worms resemble a whip the anterior three fifths being hair-like in appearance and the remaining part of the body being somewhat thicker. They have an average length of 4.5 cms and are about 2 mm in breadth at the posterior part. The eggs are barrel-shaped with a plug at each end and measure about 50 microns in length.

195. *Trichinella spiralis*—This worm is almost invisible to the naked eye being hardly 2 mm in length. The worm is viviparous and the liberated larvae enter the circulation direct and encyst in muscles. The diagnosis is difficult as no eggs are passed in the

faeces, and evidence of infection depends upon the demonstration of the parasite in muscle biopsy. Very rarely the larvae may be demonstrated in the blood or C.S.F.

196. *Examination of faeces for ova of nematodes*

Direct smear—A small portion of faeces is picked up on a wire loop and emulsified in a drop of normal saline on a microscope slide and a reasonably thick smear is made. This is then covered with a vaseline ringed cover-slip and examined first under low power and then, if necessary, under high power. This method will suffice if ova are present in at least moderate numbers, but failing this, concentration methods must be carried out. In strongyloids infection it is the larvae not ova, that are found in the faeces.

Concentration Methods

Concentration of ova in stools can be carried out either by floatation or sedimentation.

Floatation Techniques

A piece of faeces about the size of a pea is completely emulsified with saturated sodium chloride solution in a penicillin bottle. More solution is added by means of a pipette to fill the bottle right to the top. A slide is placed over the mouth of the bottle so that it has contact with the fluid. After five minutes the slide is removed vertically with care and then reversed so that the fluid from the bottle comes on the top. A cover slip is placed over the fluid on the slide and examined. The floatation technique used in the concentration of protozoal cysts can also be used for concentrating ova of helminths.

Sedimentation Technique

About 5 to 10 grams of faeces is emulsified with ten times its volume of a solution of 0.5% glycerine in tap water, and allowed to stand in a measuring cylinder for one hour. At the end of this time the upper four fifths of the solution is decanted and replaced with an equal volume of the above solution and shaken again, and

allowed to stand for half an hour. The process is repeated until the supernatant is clear. The supernatant is then completely decanted and the deposit is transferred to a slide and examined.

Method for detecting ova of E. vermicularis

The ova of Enterobius vermicularis are discharged on the perianal skin and are not found in the faeces, therefore, methods other than by examination of the faeces are necessary for its detection.

(a) *Scrapings from perianal skin*—A drop of saline is placed on a slide and the material obtained by scraping the skin round the anus is emulsified with it. A coverslip is placed on it and the slide examined.

(b) *Scotch Tape Method*—A small strip of Scotch tape or cellotape is pressed with the sticky side against the perianal skin and then lifted off and placed on a clean glass slide. The slide is examined direct.

Somatic Nematodes

Wuchereria bancrofti and malayi and Dracunculus medinensis cause somatic infection. The larval forms of Ascaris lumbricoides, Ankylostoma duodenale, Strongyloides stercoralis and Trichinella Spiralis also lead a somatic existence.

197. *Wuchereria bancrofti*—The adult worms are hair-like in appearance and measure about 3 cms in the case of the male to 8 cms in the case of the female. Both sexes live coiled together in lymph nodes, or abdominal lymphatic vessels. Their presence can be determined by lymph node biopsy. These worms, however discharge their embryos or microfilaria into the peripheral blood where they are seen to resemble minute snake-like organisms measuring about 300 microns in length. It seems to be encased in a structureless sheath which projects beyond both ends of the embryo. The microfilaria have also been demonstrated in urine, lymph varix and hydrocele fluid.

Demonstration of microfilaria in blood

198. Blood should preferably be collected between 10 pm and 2 am as microfilaria show a nocturnal periodicity. The microfilaria can be demonstrated by direct method or by concentration technique.

1. *Unstained preparation*—A drop of blood is collected on a clean slide and covered with a vaseline ringed coverslip. Wriggling microfilaria can usually be demonstrated in positive cases by examination under low power.

2. *Stained preparation*—A thick blood film prepared in exactly the same way as for malaria parasites and stained with JSB, Leishman or Geimsa's stain.

Methylene Blue Stain—A thick blood film prepared as for malaria is immersed for 5 to 10 minutes in a Koplin Jar containing 0.5% methylene blue. The slide is carefully washed in tap water, dried and examined. During the process of staining the blood film is simultaneously dehaemoglobinised. The stain appears to improve with age, more so in the presence of traces of haemoglobin.

3 *Concentration Methods.*

i. *Laked blood*—About 1 ml of blood is collected in 10 ml of distilled water or 2% formalin in a centrifuge tube and centrifuged at 2,500 r.p.m. for 5 minutes. The deposit is spread out on a slide and allowed to dry. It is then fixed with alcohol for 10 minutes and then stained as for a thick blood film and examined for microfilaria.

ii. *Citrated blood*—About 5 ml of blood is added to 1 ml of 2.5% citrated saline and centrifuged at 1,500 r.p.m. for 10 to 15 minutes. With a pasteur pipette remove a drop of the red cell deposit at the bottom of the tube and place on a slide, cover with a coverslip and examine for active microfilaria.

Identification of microfilaria

199. Sheathed microfilaria that may be found in the peripheral blood are W. bancrofti, W. malayi or Loa

loa. Their characters are sufficiently differentiated for identification.

i. Microfilaria bancrofti has an average length of about 270 microns. It has smooth curves and its tail which tapers to a point has no terminal nuclei. The cephalic space is as long as it is broad.

ii. Microfilaria malayi is about 200 microns in length and has a kinked irregular outline. The tail shows the semblance of a bulb and there are two terminal nuclei. The cephalic space is about twice as long as it is broad.

iii. Loa loa is about the same size as M. bancrofti but has the irregular kinked outline of M. malayi. The tail tapers gradually and the nuclei extend right to the tip of the tail. This does not occur in India.

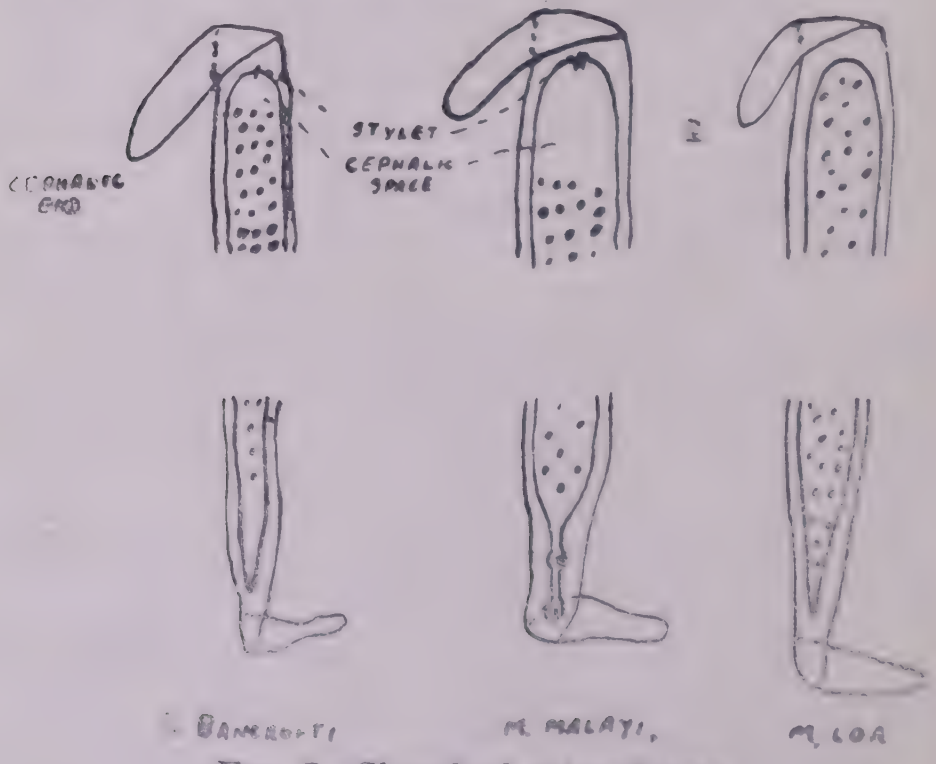

FIG. 7—Sheathed microfilaria

Dracunculus medinensis

200. The male worms of this species do not appear to have been recovered except in one or two cases. The female which occupies an irregular tortuous position in the subcutaneous tissues of the arms, legs or back, is about 1 meter in length but only 1 mm or so in diameter. The gravid uterus is packed to capacity with millions of coiled up embryos. The presence of the worm causes a blister in the skin through which the embryos are liberated when the skin comes in contact with water.

The embryos or larvae, also referred to as microfilaria are flat with round heads and long tapering tails. They measure about 600 microns in length by about 20 microns in breadth. They appear to have a jerky tadpole like movement.

201. *Diagnosis of Dracunculosis*

i. Detection of the adult worm in the skin.

ii. *Detection of embryos*—The body of the worm usually protrudes through a small bleb or ulcer. A few drops of sterile water should be placed over this area to encourage the escape of embryos from the uterus. After a few minutes a drop of fluid is withdrawn with a pasteur pipette on to a slide, covered with a coverslip and examined under high power.

Cestodes

202. These worms are, segmented and flattened and vary in size from a few millimeters to many meters. They consist of a head, neck and body or trunk called a strobila which consists of several segments, each segment being referred to as a proglottidis. There are no separate sexes, each worm being a hermaphrodite and each segment being regarded as a single individual. The adult worms are exclusively found in the intestines of man and animals. The larval forms of Taenia solium and Echinococcus granulosus can also lead a somatic existence in man in which case man is regarded as the intermediate host.

203. *Taenia solium* measures about 2 to 3 meters in length and has about 800 segments. The head is less than 1 mm in diameter, globular in appearance, has four suckers and a rostellum with a double row of hooklets. The uterus has about 8 to 10 branches on each side. The eggs are slightly brown in colour, round and about 40 microns in diameter. The shell of the ova has a radially striated covering called the embryophore containing a six-hooked embryo.

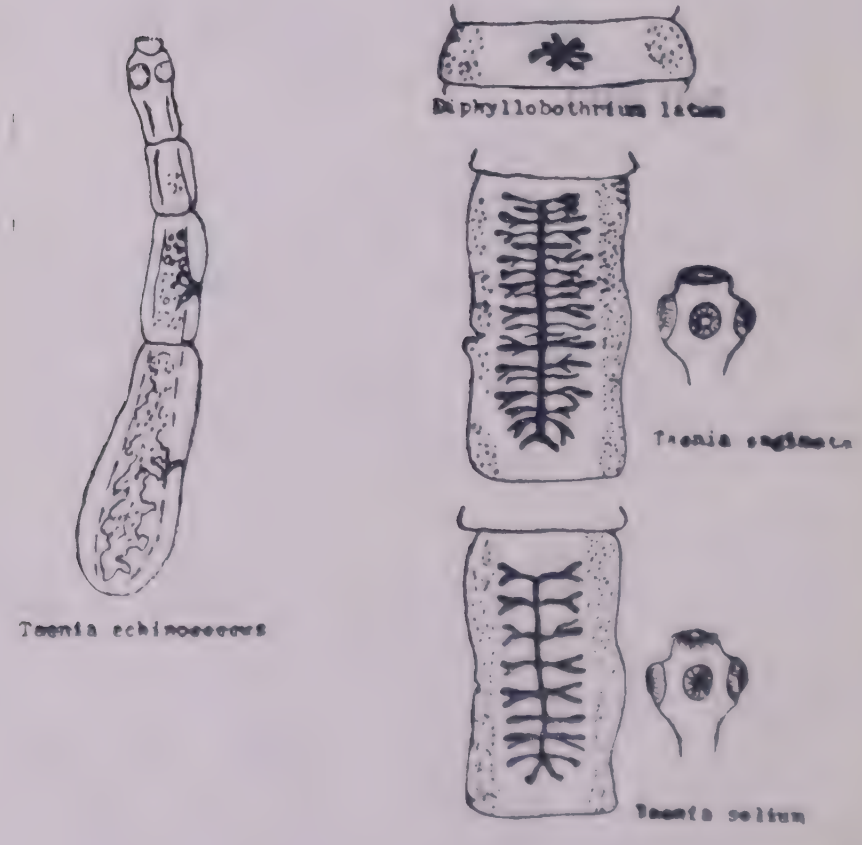

FIG. 8

204. *Taenia saginata* is somewhat longer than T. solium being about 3 or 4 meters in length and possessing about 1000 segments. The head does not have any hooklets and the uterus has about 15 to 30 lateral branches on each side. The ova are indistinguishable from those of T. solium.

205. *Hymenolepis nana* is usually found in rats and mice but may also be found in man. It is about 2 to 4 cms in length and has from 100 to 200 segments, the breadth of which is quarter of the length. The head has row of hooklets. The eggs are slightly oval and transparent. and vary from 30 to 60 microns in diameter. The six hooked embryo appears to be surrounded by two membranes; an inner embryophore which is about 20 microns thick and encloses the embryo and an outer semifluid layer about 30 microns thick limited externally by a thin shell.

206. *Diphyllobothrium latum* or *Fish Tapeworm* is the longest of the tapeworms and measures up to 4 meters in length and has about 3000 to 4000 segments. The head is about 2 to 3 mm in length and has two suckers in the form of grooves. The segments are broader than they are long. The eggs are operculated and measure 70 microns in length by about 40 microns in breadth and contain an unsegmented ovum which develops in water into a hexacanth embryo. It does not occur in India.

Examination of faeces for ova of cestodes

207. The direct smear is prepared and examined in exactly the same way as for nematode eggs.

In carrying out concentration methods it must be pointed out that the specific gravity of eggs of Taenia are greater than the specific gravity of saturated salt solution (.S.G. 1200) and therefore the eggs cannot be concentrated by the floatation technique. Sedimentation is the method of choice for concentrating eggs of taenia. Eggs of cestoda other than Taenia can be concentrated by any method described under Nematoda.

Identification of segments of cestoda

208. As the identification of the segments of the tapeworms depends on the demonstration of the uterine branches, the segment must first be rendered transparent by placing the segment in a watchglass containing pure carbolic and left there for an hour. At the end of this time the segment is picked up with forceps and washed quickly with water to get rid of excess carbolic acid and then pressed slightly between two slides and held to the light. The uterine branches at once become apparent and can be counted to differentiate between T. solium and T. saginata. For more detailed examination, the segment can be examined under the dissecting microscope.

209. *Echinococcus granulosus*—Infection with adult worm occurs primarily in dogs and allied animals but not in man. Man, however, is the intermediate host in the life history of this worm in whom the larval form gives rise to hydatid disease. The adult worm is barely 0.5 cm in length and occurs in thousands in the intestines of infected dogs. It has a head, neck and strobila of 3 or 4 segments. The egg is about 35 microns in diameter and resembles the eggs of Taenia. It is infective to man and other herbivorous animals like sheep and cattle.

Only the larval form of the parasite produces hydatid disease in man, the ova and the adult worm are only found in dogs, foxes and wolves.

Diagnosis of Hydatid cyst is mainly clinical, but the following laboratory methods are of help in establishing the diagnosis.

i. Examination of sputum or material obtained by puncture will reveal scolices or pieces of laminated membrane from the cyst.

i. Immunological tests such as the complement fixation test and Casoni's intra-dermal test in which hydatid fluid is used as the antigen. These are dealt with under the section "Serology".

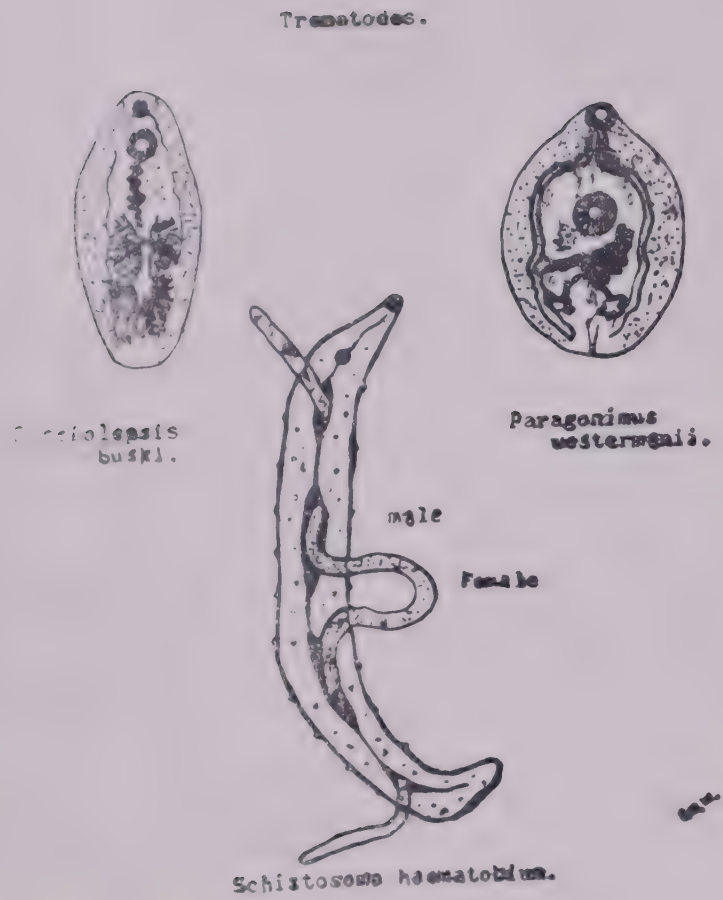

FIG. 9—Trematodes

Trematodes

210. These are so named because of their conspicuous suckers. They vary in size from 1 mm to several centimetres. Many are covered with spines, ridges or tubercles. All these worms are hermaphrodites except schistosomes. They are classified according to their habitat viz. Intestinal Flukes, Liver Flukes, Lung Flukes and Blood Flukes. Except for Fasciolopsis buski

which is an intestinal fluke and reported from Bengal and Assam. Paragonimus westermanii which is a lung fluke and has been reported from Bengal, Assam and Madras and S. haematobium which has been reported from Ratnagiri Distt in India, others are not found in India.

211. *Fasciolopsis buski*—This is the largest of the trematodes and measures 7 cms by about 2 cms. It is oval in shape with a cone-shaped anterior end and a large ventral sucker about 3 mm in diameter. The body is covered with spines. The eggs are operculated and measure 140 microns by 80 microns; they contain a large unsegmented ovum in a mass of yolk cells.

212. *Paragonimus westermanii*—These worms measure about 1 cm by 5 mm and in shape they resemble one half of a pea. They are fleshy and of a reddish brown colour with a prominent ventral sucker. The eggs are of a light brown colour oval in shape and measure 80 microns by 45 microns. The operculated side is flattened. These eggs are mainly found in the sputum but they may be found in the faeces of an infected person.

213. *Schistosoma haematobium*—The sexes are separate and both provided with suckers. The male is about 1 cm by 1 mm. and on both sides the body is folded ventrally to form a groove called the gynaecophoric canal. The outer surface of the worm is finely tuberculated. The female is 2 cms long and is fine hair-like in appearance. The middle portion of the female worm is enclosed in the ventral groove of the male. The eggs are elongated and spindle shaped and measure about 145 microns in length by about 45 microns in breadth. At one end of the egg is a short terminal spine. As this worm usually selects the vesical plexus for its habitat, the diagnosis of this infection is based upon the demonstration of the characteristic terminal-spined egg in the urine.

214. *Schistosoma mansoni*—Morphologically this worm resembles S. haematobium but its outer surface

is grossly tuberculated compared with the fine tubercles of S. haematobium. The eggs are about the same size as S. haematobium, are oval in shape and possess a sharp lateral spine. This worm selects the haemorrhoidal plexus for its habitat, it therefore causes intestinal symptoms and the eggs are found in the faeces.

215. *Schistosoma japonicum*—Morphologically this worm also resembles S. haematobium except that its outer surface is smooth. The eggs are slightly oval and measure about 85 microns by 65 microns, and have a minute hook or spine. Like S. mansoni the eggs are found in the faeces.

216. *Examination of faeces*

Direct smear—This is prepared in the same way as for other helminthic ova.

Concentration method—Operculate ova are not adapted to floatation and therefore concentration of ova can only be carried out by sedimentation. Since schistosome eggs tend to hatch out in five hours in presence of water, the examination of stools should be complete within this time.

217. *Examination of urine*

The urine for examination for schistosome eggs should be collected at the end of micturition. This urine is then centrifuged at about 1500 r.p.m. for five minutes and the deposit removed by means of a capillary pipette. A drop of it is placed on a slide, covered with a coverglass and examined.

218. *Examination of sputum for P. westermanii*

The sputum is diluted with an equal volume of 3 per cent. sodium hydroxide and allowed to incubate at 37°C for half an hour to liquify the mucus. This is then centrifuged for 10 to 20 minutes at 3000 r.p.m. and the sediment taken on a slide and examined for ova.

PRESERVATION OF MATERIAL

Protozoal cysts and ova of helminths in faeces

219. About a gram of faeces is mixed with 2 to 3 ml of tap water and thoroughly emulsified. About 50 ml of 10 per cent. formalin heated to 90°C is poured on to the emulsified faeces which are kept stirred during the process. After standing for 8 to 10 hours

1.Ascaris lumbricoides.2.Ankylostoma duodenale.3.Ancylostoma caninum.4.Trichuris trichiura.5.Strongyloides stercoralis.6.Hymenolepis nana.7.Taenia solium.8.Hymenolepis nana.9.Schistosoma japonicum.10.Schistosoma haematobium.11.Schistosoma mansoni. 12.Diphyllobothrium latum.

FIG. 10

the supernatant is decanted and replaced with 5 per cent formalin.

220. **Helminths**

Nematodes—These worms should be removed from the faeces and washed in normal saline and then dropped into 5 per cent. formalin heated to 70°.C to kill them. They are then preserved in 75 per cent. alcohol to which 40 per cent. glycerine has been added.

Cestodes—If found in the intestines at post mortem they should be removed carefully with that portion of the intestine to which the head is attached. It is then left in running tap water for one or two hours until it becomes detached from the intestines. The worm should then be wrapped round a glass plate which is placed in hot 5 per cent. formalin and then transferred to 3 per cent. formalin.

Trematodes—These worms are placed in 3 per cent. formalin. The larger flukes should preferably be placed between two pieces of glass plate and then immersed in 3 per cent formalin.

The following books are recommended for further reading on the subject of parasitology.

(1) Human parasites and parasitic diseases—K. D. CHATTERJEE.

(2) Practical Bacteriology, Haematology & Parasitology—*E. R. STITT, PAUL W. CLOUGH & SARA E BRANHAM.*

(3) A manual of Tropical medicine—*THOMAS T. MACKIE, GEORGE, W. HUNTER, C. BROOKEWORTH.*

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